

Transgenic expression cassettes for expressing nucleic acids in nonreproductive floral tissues of plants

- 5 The invention relates to methods for the targeted transgenic expression of nucleic acid sequences in nonreproductive floral tissues of plants, and to transgenic expression cassettes and expression vectors which comprise promoters having an expression specificity for nonreproductive floral tissues of plants. The  
10 invention further relates to organisms (preferably plants) transformed with these transgenic expression cassettes or expression vectors, to cultures, parts or propagation material derived therefrom, and to the use of the same for producing human and animal foods, seeds, pharmaceuticals or fine chemicals.  
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- The aim of biotechnological operations on plants is to produce plants with advantageous novel properties, for example for increasing the agricultural productivity, for increasing the  
20 quality of human foods or for producing particular chemicals or pharmaceuticals (Dunwell JM (2000) J Exp Bot 51 Spec No:487-96). A basic precondition for transgenic expression of particular genes is the provision of plant-specific promoters. Promoters are important tools in plant biotechnology for controlling the  
25 expression of particular genes in a transgenic plant and thus achieving particular traits of the plant.

- Various plant-specific promoters are known, for example constitutive promoters such as the promoter of the Agrobacterium  
30 nopaline synthase, the TR dual promoter or the promoter of the cauliflower mosaic virus (CaMV) 35S transcript (Odell et al. (1985) Nature 313:810-812). A disadvantage of these promoters is that they are constitutively active in virtually all tissues of the plant. Targeted expression of genes in particular plant  
35 parts or at particular times of development is not possible with these promoters.

- Promoters having specificities for various plant tissues such as anthers, ovaries, flowers, leaves, stalks, roots, tubers or seeds  
40 have been described. The stringency of the specificity and the expression activity of these promoters varies widely.

- The flower of plants serves for sexual reproduction of flowering plants. The flowers of plants – especially the petals –  
45 frequently accumulate large amounts of secondary plant products such as, for example, terpenes, anthocyanins, carotenoids, alkaloids and phenylpropanoids, which serve as scents, defensive

substances or as colorants in the flower. Many of these substances are of commercial interest. In addition, the flower bud and the flower of the plant is a sensitive organ, especially to stress factors such as cold.

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The *Arabidopsis thaliana* gene locus At3g01980 (GenBank Acc.-No.: NC\_003074; *Arabidopsis thaliana* chromosome 3; base pairs: complement 327677 to 329029) encodes a putative dehydrogenase (derived cDNA: GenBank Acc.-No: NM\_111064; SEQ ID NO: 11). The  
10 *Arabidopsis thaliana* gene locus At1g63140 (GenBank Acc.-No: NC\_003070.2; *Arabidopsis thaliana* chromosome 1; base pairs 23069430 to 23070871) encodes a putative caffeic acid o-methyltransferase (derived cDNA: GenBank Acc.-No: NM\_104992.1; SEQ ID NO: 13). The precise function, transcription and the  
15 expression patterns of these genes are not described.

Flower-specific promoters such as, for example, the phytoene synthase promoter (WO 92/16635), the promoter of the P-rr gene  
20 (WO 98/22593) or the promoter of the APETALA3 gene (Hill TA et al. (1998) Development 125:1711-1721) are known. However, all these promoters have one or more disadvantages which are prejudicial to wide use:

- 25 1) within the flower they are specific for one or more flower tissues and do not guarantee expression in all tissues of the flower.
- 30 2) they are – as in the example of the APETALA3 gene which is involved in flower development – highly regulated during flower development and are not active in all phases of flower development.
- 35 3) they occasionally show strong secondary activities in other plant tissues. Thus, the known promoters (such as, for example, the APETALA3 promotor) show in most cases an activity in seeds, anthers and the ovaries of the flower, which constitute sensitive floral organs which are directly  
40 involved in the plants' reproduction. Expression here is in many cases unnecessary and disadvantageous since it may interfere with the plants' reproduction. Moreover, the expressed gene product can be dispersed in an undesired manner by seeds and pollen in the air. For the purposes of a  
45 biotechnological exploitation of transgenic plants, this is largely to be avoided.

Despite the large number of known plant promoters, no promoter with a specificity for the plant flower which essentially lacks expression in the pollens and ovaries, i.e. which is only active in the nonreproductive tissues, has been identified to date, nor  
5 are any promoters known which, in addition to having the abovementioned specificity, are active essentially during all of the floral development.

It is therefore an object to provide methods and suitable  
10 promoters for the targeted, transgenic expression of nucleic acids into the nonreproductive floral tissues. We have found that this object is achieved by providing the promoters of the genes with the gene locus names At3g01980 (hereinbelow "76L" promoter; SEQ ID NO: 1) and At1g63140 (hereinbelow "84L" promoter; SEQ ID  
15 NO: 2) from *Arabidopsis thaliana*. These promoters show expression in all floral organs with the exception of the pollen and the ovaries. This expression pattern can be observed in the flower bud, the flower and the wilting flower.

20 A first aspect of the invention relates to methods for the targeted transgenic expression of nucleic acid sequences in nonreproductive floral tissues of plants, comprising the following steps:

- 25 I. introduction of a transgenic expression cassette into plant cells, wherein the transgenic expression cassette comprises at least the following elements
- 30 a) at least one promoter sequence selected from the group of sequences consisting of
- i) the promoter sequences of SEQ ID NO: 1 or 2 and
- 35 ii) functional equivalents of the promoter sequences of SEQ ID NO: 1 or 2 with essentially the same promoter activity as a promoter of SEQ ID NO: 1 or 2 and
- 40 iii) functional equivalent fragments of the sequences of i) or ii) with essentially the same promoter activity as a promoter of SEQ ID NO: 1 or 2,
- and
- 45 b) at least one further nucleic acid sequence, and

c) optionally further genetic control elements,

5 wherein at least one promoter sequence and one further nucleic acid sequence are functionally linked together, and the further nucleic acid sequence is heterologous in relation to the promoter sequence, and

10 II. selection of transgenic cells which comprise said expression cassette stably integrated into the genome, and

15 III. regeneration of complete plants from said transgenic cells, wherein at least one of the further nucleic acid sequences is expressed essentially in all nonreproductive floral tissues, but essentially not in the pollen and the ovaries.

A further aspect relates to transgenic expression cassettes as, for example, can be employed in the method of the invention. The  
20 transgenic expression cassettes preferably comprise for the targeted transgenic expression of nucleic acid sequences in nonreproductive floral tissues of plants,

25 a) at least one promoter sequence selected from the group of sequences consisting of

i) the promoter sequences of SEQ ID NO: 1 or 2 and

30 ii) functional equivalents of the promoter sequences of SEQ ID NO: 1 or 2 with essentially the same promoter activity as a promoter of SEQ ID NO: 1 or 2 and

35 iii) functionally equivalent fragments of the sequences of i) or ii) with essentially the same promoter activity as a promoter of SEQ ID NO: 1 or 2,

and

40 b) at least one further nucleic acid sequence, and

c) optionally further genetic control elements,

45 wherein at least one promoter sequence and one further nucleic acid sequence are functionally linked together, and the further nucleic acid sequence is heterologous in relation to the promoter sequence.

The expression cassettes of the invention may comprise further genetic control sequences and/or additional functional elements.

5 It is possible and preferred for the transgenic expression cassettes to make possible, through the nucleic acid sequence to be expressed transgenically, the expression of a protein encoded by said nucleic acid sequence and/or the expression of a sense-RNA, antisense-RNA or double-stranded RNA encoded by said nucleic acid sequence.

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A further aspect of the invention relates to transgenic expression vectors which comprise one of the expression cassettes of the invention.

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A further aspect of the invention relates to transgenic organisms which comprise one of the expression cassettes or expression vectors of the invention. The organism can be selected from the group consisting of bacteria, yeasts, fungi, nonhuman animal and  
20 plant organisms or of cells, cell cultures, parts, tissues, organs or propagation material derived therefrom, and the organism is preferably selected from the group of agricultural crop plants.

25 A further aspect of the invention therefore relates to the use said transgenic organisms or cells, cell cultures, parts, tissues, organs or propagation material derived therefrom to produce human and animal foods, seeds, pharmaceuticals or fine chemicals, where the fine chemicals are preferably enzymes,  
30 vitamins, amino acids, sugars, saturated or unsaturated fatty acids, natural or synthetic flavorings, aromatizing substances or colorants. The invention further includes methods for producing said human and animal foods, seeds, pharmaceuticals or fine chemicals employing the transgenic organisms of the invention or  
35 cells, cell cultures, parts, tissues, organs or propagation material derived therefrom.

The transgenic expression cassettes of the invention are particularly advantageous for the following reasons:  
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a) they impart selective expression in nonreproductive tissues of the flower bud and the flower of plants and make numerous applications possible, such as, for example, resistance to  
45 stress factors such as cold or targeted synthesis of

secondary plant products. Expression takes place throughout the period of flower bud and flower development.

- b) they show no expression in reproductive tissues (such as pollen or ovaries), whereby interference with the reproduction and spreading of the transgenic protein by pollen or seeds in the air is avoided.

The transgenic expression cassettes according to the invention, and the transgenic expression vectors and transgenic organisms derived from them, may comprise functional equivalents of the promoter sequences described under SEQ ID NO: 1 or 2.

The promoter activity of a functionally equivalent promoter is referred to as being "essentially the same" when the transcription of a particular nucleic acid sequence to be expressed transgenically under the control of said functionally equivalent promoter under otherwise unchanged conditions shows a targeted expression in essentially all nonreproductive floral tissues, with essentially no expression in the pollen and ovaries.

"Flower" generally means a shoot of limited growth whose leaves have been transformed into reproductive organs. The flower consists of various "floral tissues" such as, for example, the sepals, the petals, the stamens or the carpels. Androecium is the term used for the totality of stamens in the flower. The stamens are located within the circle of petals and sepals. A stamen is composed of a filament and of an anther located at the end. The latter in turn is divided into two thecae which are connected together by a connective. Each theca consists of two pollen sacs in which the pollen is formed.

"Nonreproductive floral tissue" refers to all tissues of the flower except for the pollen and the ovaries.

"Essentially all nonreproductive floral tissues" means with regard to the nonreproductive floral tissues that some of these tissues, either in total or at specific points in time of their development, may display no substantial expression, where these tissues however amount to preferably less than 20% by weight, preferably less than 10% by weight, especially preferably less than 5% by weight, very especially preferably less than 1% by weight of the total weight of the nonreproductive floral tissues.

"Targeted" means in relation to the expression in nonreproductive

floral tissues preferably that the expression under the control of one of the promoters of the invention in the nonreproductive floral tissues is at least ten times, particularly preferably at least fifty times, very particularly preferably at least one hundred times that of another tissue such as, for example, the pollen or the ovaries or a nonfloral tissue, such as the leaves.

The fact that the promoters according to the invention "show essentially no expression in the pollen and ovaries" preferably means that the statistical mean of the expression over all reproductive floral tissues is at most 10%, preferably at most 5%, most preferably at most 1% that of the statistical mean of the expression over all nonreproductive floral tissues under identical conditions.

Preferably, the expression within the nonreproductive floral tissues is essentially constant. "Essentially constant" in this context preferably means that the standard deviation of the expression between the individual nonreproductive floral tissues relative to the statistical mean of the expression over all nonreproductive floral tissues is less than 50%, preferably 20%, especially preferably 10%, very especially preferably 5%.

Preferably, the expression within at least one particular nonreproductive floral tissue is essentially constant over all developmental stages of the flower. "Essentially constant" in this context preferably means that the standard deviation of the expression between the individual points in time of the development of the particular nonreproductive floral tissue relative to the statistical mean of the expression over all points in time of the development is less than 50%, preferably 20%, especially preferably 10%, very especially preferably 5%.

The nucleic acid sequences in functional linkage with the test promoter which are preferably employed for estimating the level of expression are those which code for easily quantifiable proteins. Very particular preference is given in this connection to reporter proteins (Schenborn E, Groskreutz D. (1999) Mol Biotechnol 13(1): 29-44) such as the green fluorescence protein (GFP) (Chiu WL et al. (1996) Curr Biol 6:325-330; Leffel SM et al. (1997) Biotechniques 23(5):912-8), chloramphenicol transferase, luciferase (Millar et al. (1992) Plant Mol Biol Rep 10:324-414),  $\beta$ -glucuronidase or  $\beta$ -galactosidase. Very particular preference is given to  $\beta$ -glucuronidase (Jefferson et al. (1987) EMBO J 6:3901-3907).

"Conditions which are otherwise unchanged" means that the expression initiated by one of the transgenic expression cassettes to be compared is not modified by combination with additional genetic control sequences, for example enhancer  
5 sequences. Unchanged conditions further means that all general conditions such as, for example, plant species, stage of development of the plants, culture conditions, assay conditions (such as buffer, temperature, substrates etc.) are kept identical between the expressions to be compared.

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"Transgenic" means — for example in relation to an expression cassette (or to an expression vector or transgenic organism comprising the former) all those constructions which have originated by recombinant methods in which either

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- a) the promoter of SEQ ID NO: 1 or 2 or a functional equivalent thereof or a functional equivalent part of the above, or
- 20 b) a further nucleic acid sequence which is functionally linked with a), or
- c) (a) and (b)

- 25 are not in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to be, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The promoter sequence of the invention (e.g. the  
30 sequence as shown in SEQ ID NO: 1, 2, 3 or 4) comprised in the expression cassettes is preferably heterologous in relation to the further nucleic acid sequence which is to be expressed transgenically and is functionally linked thereto. "Heterologous" means in this connection that the further nucleic acid sequence  
35 does not code for the gene which is naturally under the control of said promoter.

- "Natural genetic environment" means the natural chromosomal locus  
40 in the original organism or the presence in a genomic library. In the case of a genomic library, the natural, genetic environment of the nucleic acid sequence is preferably still retained at least in part. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp,  
45 preferably at least 500 bp, particularly preferably at least 1000 bp, very particularly preferably at least 5000 bp. A naturally occurring expression cassette — for example the naturally occurring combination of the promoter of a gene coding



for a protein as shown in SEQ ID NO: 12 or 14 or a functional equivalent thereof with its corresponding coding sequences becomes a transgenic expression construct when the latter is modified by unnatural, synthetic ("artificial") methods such as, 5 for example, a mutagenesis. Appropriate methods are described (US 5,565,350; WO 00/15815; see also above).

"Transgenic" means in relation to an expression ("transgenic expression") preferably all expressions caused by use of a 10 transgenic expression cassette, transgenic expression vector or transgenic organism - complying with the definitions given above.

Functional equivalents of a promoter of SEQ ID NO: 1 or 2 in 15 particular means natural or artificial mutations of a promoter of SEQ ID NO: 1 or 2 and homologous sequences from other organisms, preferably from plant organisms, which have essentially the same promoter activity as one of the promoters of SEQ ID NO: 1 or 2..

20 Functional equivalents also comprises all those sequences which are derived from the complementary counterstrand of the sequences defined by SEQ ID NO: 1 or 2 and have essentially the same promoter activity.

25 Functional equivalents to the promoters of SEQ ID NO: 1 or 2 preferably comprise those sequences which

- 30 a) have essentially the same promoter activity as one of the promoters of SEQ ID NO: 1 or 2 and
- b) have a homology of at least 50%, preferably 70%, more preferably at least 80%, particularly preferably at least 90%, very particularly preferably at least 95%, most 35 preferably 99%, with the sequence of one of the promoters of SEQ ID NO: 1 or 2, wherein the homology extends over a length of at least 100 base pairs, preferably at least 200 base pairs, particularly preferably of at least 300 base pairs, very particularly preferably of at least 400 base pairs, most 40 preferably of at least 500 base pairs.

It is possible in this connection for the level of expression of the functional equivalents to differ both downwards and upwards from a comparison value. Preference is given in this connection 45 to the sequences whose level of expression, measured on the basis of the transcribed mRNA or the subsequently translated protein, under conditions which are otherwise unchanged differs

quantitatively by not more than 50%, preferably 25%, particularly preferably 10%, from a comparison value obtained with the promoters described by SEQ ID NO: 1 or 2. Particularly preferred sequences are those whose level of expression, measured on the basis of the transcribed mRNA or the subsequently translated protein, under conditions which are otherwise unchanged exceeds quantitatively by more than 50%, preferably 100%, particularly preferably 500%, very particularly preferably 1000%, a comparison value obtained with the promoter described by SEQ ID NO: 1 or 2.

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Examples of promoter sequences employed in the transgenic expression cassettes or transgenic expression vectors of the invention can easily be found for example in various organisms whose genomic sequence is known, such as, for example, Arabidopsis thaliana, Brassica napus, Nicotiana tabacum, Solanum tuberosum, Helianthium annuus, Linum sativum, by making homology comparisons in databases. A possible and preferred starting point for this is the coding regions of the genes whose promoters are described by SEQ ID NO: 1 or 2. Starting from, for example, the cDNA sequences of these genes described by SEQ ID NO: 11 or 13 or the protein sequences derived therefrom and described by SEQ ID NO: 12 or 14 it is possible easily to identify, in a manner familiar to the skilled worker, the corresponding homologous genes in other plant species by screening databases or gene libraries (using appropriate gene probes).

In a preferred embodiment of the invention, functional equivalents of the promoter described by SEQ ID NO: 1 comprise all those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a protein with at least 60%, preferably at least 80%, especially preferably at least 90%, most preferably at least 95% homology with the protein of SEQ ID NO: 12, wherein said promoters constitute the natural promoter of said genomic sequence. Especially preferably, functional equivalents of the promoter described by SEQ ID NO: 1 comprise all those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a nucleic acid sequence whose derived cDNA has at least 60%, preferably at least 80%, especially preferably at least 90%, most preferably at least 95% homology with the nucleic acid sequence as shown in SEQ ID NO: 11, wherein said promoters constitute the natural promoter of said genomic sequence. Preferred promoters comprise a sequence region of least 250 base pairs, preferably at least 500 base pairs, particularly preferably 1000 base pairs, most preferably at least 2000 base pairs, in the 5' direction calculated from the ATG start codon of said genomic sequences. Functional equivalents

of the promoter described by SEQ ID NO: 1 are particularly preferably all promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a protein which comprises at least one of the following sequence motifs:

1. NGD(E/Q)VSRNIA (SEQ ID NO: 23)
2. LAKHGC(R/K)LV (SEQ ID NO: 24)
3. MGNEXSLSRXVDXIR (SEQ ID NO: 25)
- 10 4. TYQGKXQDILXVS(Q/E)DEF (SEQ ID NO: 26)
5. IT(K/R)INLTAXWFXLKAVA (SEQ ID NO: 27)

Very particularly preferred functional equivalents of the promoter described by SEQ ID NO: 1 are those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a protein, wherein said protein comprises at least one of the following sequences:

1. the homologous sequence (H2) from oilseed rape as shown in SEQ ID NO: 16
2. the homologous sequence (H3) from oilseed rape as shown in SEQ ID NO: 18

Most preferred functional equivalents of the promoter described by SEQ ID NO: 1 are those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a nucleic acid sequence whose derived cDNA comprises at least one of the following sequences:

1. the homologous sequence (H2) from oilseed rape as shown in SEQ ID NO: 15
2. the homologous sequence (H3) from oilseed rape as shown in SEQ ID NO: 17

In a preferred embodiment of the invention, functional equivalents of the promoter described by SEQ ID NO: 2 comprise all those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a protein with at least 60%, preferably at least 80%, especially preferably at least 90%, most preferably at least 95% homology with the protein of SEQ ID NO: 14, wherein said promoters constitute the natural promoter of said genomic sequence. Especially preferably, functional equivalents of the promoter described by SEQ ID NO: 2 comprise all those promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a nucleic acid sequence whose

derived cDNA has at least 60%, preferably at least 80%, especially preferably at least 90%, most preferably at least 95% homology with the nucleic acid sequence as shown in SEQ ID NO: 13, wherein said promoters constitute the natural promoter of  
 5 said genomic sequence. Preferred promoters comprise a sequence region of least 250 base pairs, preferably at least 500 base pairs, particularly preferably 1000 base pairs, most preferably at least 2000 base pairs, in the 5' direction calculated from the ATG start codon of said genomic sequences. Functional equivalents  
 10 of the promoter described by SEQ ID NO: 2 are particularly preferably all promoters which are located in a plant organism in the 5' direction upstream of a genomic sequence which encodes a protein which comprises at least one of the following sequence motifs:

- 15 1. AEPVCTXFL (SEQ ID NO: 28)
2. EGKDXFXSAHGMXXFE (SEQ ID NO: 29)
3. EQFAXMFNXAM (SEQ ID NO: 30)
4. ATXIMKK(V/I)LEVY(K/R)GFED (SEQ ID NO: 31)
- 20 5. TLVD(V/I)GGGXGT (SEQ ID NO: 32)

Very particularly preferred functional equivalents of the promoter described by SEQ ID NO: 2 are those promoters which are located in a plant organism in the 5' direction upstream of a  
 25 genomic sequence which encodes a protein, wherein said protein comprises at least one of the following sequences:

1. the homologous sequence (H4) from oilseed rape as shown in SEQ ID NO: 20
- 30 2. the homologous sequence (H5) from oilseed rape as shown in SEQ ID NO: 22

Most preferred functional equivalents of the promoter described by SEQ ID NO: 2 are those promoters which are located in a plant  
 35 organism in the 5' direction upstream of a genomic sequence which encodes a nucleic acid sequence whose derived cDNA comprises at least one of the following sequences:

- 40 1. the homologous sequence (H4) from oilseed rape as shown in SEQ ID NO: 19
2. the homologous sequence (H5) from oilseed rape as shown in SEQ ID NO: 21

45 A further subject of the invention therefore relates to polypeptides comprising an amino acid sequence as shown in SEQ ID NO: 16, 18, 20 or 22 and the nucleic acid sequences encoding

them, preferably the sequences comprising a sequence as shown in SEQ ID NO: 15, 17, 19 or 21 or the sequences derived therefrom the result of degeneracy of the genetic code.

- 5 A further aspect of the invention relates to the use of at least one nucleic acid sequence or of a part thereof in methods for identifying and/or isolating promoters of genes which code for said nucleic acid sequence, wherein said nucleic acid sequence encodes an amino acid sequence comprising at least one sequence  
10 as shown in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 or a variation indicated for these sequences. Said nucleic acid sequence preferably codes for an amino acid sequence comprising a sequence as shown in SEQ ID NO: 12, 14, 16, 18, 20 or 22. Said nucleic acid sequence particularly preferably comprises a  
15 sequence as shown in SEQ ID NO: 11, 13, 15, 17, 19 or 21. "Part" means in relation to the nucleic acid sequence preferably a sequence of at least 10 bases, preferably 15 bases, particularly preferably 20 bases, most preferably 30 bases.
- 20 Further included according to the invention are methods for identifying and/or isolating promoters of genes which encode a promoter having specificity for nonreproductive floral tissue, wherein at least one nucleic acid sequence or a part thereof is  
25 employed in the identification and/or isolation, wherein said nucleic acid sequence encodes an amino acid sequence which comprises at least one sequence as shown in SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 or a variation indicated for these sequences. Said nucleic acid sequence preferably codes for  
30 an amino acid sequence comprising a sequence as shown in SEQ ID NO: 12, 14, 16, 18, 20 or 22. Said nucleic acid sequence particularly preferably comprises a sequence as shown in SEQ ID NO: 11, 13, 15, 17, 19 or 21. "Part" means in relation to the nucleic acid sequence preferably a sequence of at least 10 bases,  
35 preferably 15 bases, particularly preferably 20 bases, most preferably 30 bases. In a preferred embodiment, the method of the invention is based on the polymerase chain reaction, wherein said nucleic acid sequence or a part thereof is employed as primer.
- 40 Various methods for identifying and isolating the promoter of the corresponding gene, starting from a nucleic acid sequence (e.g. a gene transcript such as, for example, a cDNA) are known to the skilled worker. In principle, all methods for amplifying flanking chromosomal sequences are available for example for this purpose.
- 45 The two most commonly used methods are inverse PCR ("iPCR"; diagrammatically depicted in Fig. 10) and "Thermal Asymmetric Interlaced PCR" ("TAIL PCR").

For the iPCR, genomic DNA of the organism from which the functionally equivalent promoter is to be isolated is completely digested with a given restriction enzyme, and then the individual fragments are religated, i.e. linked to themselves to give a  
5 circular molecule, in a diluted mixture. The large number of resulting circular DNA molecules also includes those comprising the known sequence (for example the sequence coding for the homologous protein). Starting from this, the circular molecule can be amplified by PCR using a primer pair where both primers  
10 are able to anneal to the known sequence segment. One possible embodiment of the iPCR is reproduced in example 6.

The TAIL-PCR is based on the use of firstly a set of successively truncated highly specific primers which anneal to the known  
15 genomic sequence (for example the sequence coding for the homologous protein), and secondly a set of shorter random primers with a lower melting temperature, so that a less sequence-specific annealing to genomic DNA flanking the known genomic sequence takes place. Annealing of the primers to the DNA  
20 to be amplified is possible with such a primer combination making specific amplification of the desired target sequence possible. One possible embodiment of the TAIL-PCR is reproduced for example in example 5.

25 A further aspect of the invention relates to methods for producing a transgenic expression cassette having specificity for nonreproductive floral tissue, comprising the following steps:

30 I. isolation of a promoter sequence with specificity for nonreproductive floral tissues, wherein at least one nucleic acid sequence or a part thereof is employed in the isolation, wherein said nucleic acid sequence encodes an amino acid sequence which comprises at least one sequence as shown in  
35 SEQ ID NO: 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32 or a variation indicated for these sequences.

II. functional linkage of said promoter with a further nucleic acid sequence, wherein said nucleic acid sequence is  
40 heterologous in relation to the promoter.

Said nucleic acid sequence preferably codes for an amino acid sequence comprising a sequence as shown in SEQ ID NO: 12, 14, 16, 18, 20 or 22. Said nucleic acid sequence particularly preferably  
45 comprises a sequence as shown in SEQ ID NO: 11, 13, 15, 17, 19 or 21. "Part" means in relation to the nucleic acid sequence preferably a sequence of at least 10 bases, preferably 15 bases,

particularly preferably 20 bases, most preferably 30 bases. In a preferred embodiment, the method of the invention is based on the polymerase chain reaction, wherein said nucleic acid sequence or a part thereof is employed as primer. Methods known to the skilled worker, such as, for example, ligation etc., can be employed for the functional linkage (see below).

"Mutation" means substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Thus, for example, the present invention also comprises nucleotide sequences obtained by modification of the promoters as shown in SEQ ID NO: 1 or 2. The aim of such a modification may be further localization of the sequence comprised therein or, for example, also the insertion of further restriction enzyme cleavage sites, the deletion of excess DNA or the addition of further sequences, for example further regulatory sequences.

Where insertions, deletions or substitutions, such as, for example, transitions and transversions, are appropriate, it is possible to use techniques known per se, such as in vitro mutagenesis, primer repair, restriction or ligation. Transition means a base-pair exchange of a purine/pyrimidine pair into another purine/pyrimidine pair (e.g. A-T for G-C). Transversion means a base-pair exchange of a purine/pyrimidine pair for a pyrimidine/purine pair (e.g. A-T for T-A). Deletion means removal of one or more base pairs. Insertion means introduction of one or more base pairs.

Complementary ends of the fragments for ligation can be made available by manipulations such as, for example, restriction, chewing back or filling in of overhangs for blunt ends. Analogous results are also obtainable by using the polymerase chain reaction (PCR) using specific oligonucleotide primers.

Homology between two nucleic acids means the identity of the nucleic acid sequence over the complete sequence length in each case, which is calculated by comparison with the aid of the GAP program algorithm (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA), setting the following parameters:

Gap Weight: 12

Length Weight: 4

Average Match: 2.912

Average Mismatch:-2.003

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For example, a sequence which has a homology of at least 50% based on nucleic acids with the sequence SEQ ID NO: 1 means a sequence which has a homology of at least 50% on comparison with the sequence SEQ ID NO: 1 by the above program algorithm with the 5 above set of parameters.

Homology between two polypeptides means the identity of the amino acid sequence over the respective sequence length, which is calculated by comparison with the aid of the GAP program 10 algorithm (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA), setting the following parameters:

15	Gap Weight: 8	Length Weight: 2
	Average Match: 2.912	Average Mismatch:-2.003

For example, a sequence having a homology of at least 60% based 20 on protein with the sequence SEQ ID NO: 12 means a sequence which has a homology of at least 60% on comparison with the sequence SEQ ID NO: 12 by the above program algorithm with the above set of parameters.

25 Functional equivalents also means DNA sequences which hybridize under standard conditions with one of the nucleic acid sequences coding for one of the promoters as shown in SEQ ID NO: 1 or 2 or with the nucleic acid sequences complementary thereto, and which 30 have substantially the same promoter properties.

The term standard hybridization conditions is to be understood broadly and means both stringent and less stringent hybridization conditions. Such hybridization conditions are described inter 35 alia in Sambrook J, Fritsch EF, Maniatis T et al., in Molecular Cloning - A Laboratory Manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press, 1989, pages 9.31-9.57 or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the conditions during the washing step 40 can be selected from the range of conditions limited by those of low stringency (with approximately 2X SSC at 50°C) and those of high stringency (with approximately 0.2X SSC at 50°C, preferably at 65°C) (20X SSC: 0.3 M sodium citrate, 3 M NaCl, pH 7.0). In addition, the temperature during the washing step can be raised 45 from low-stringency conditions at room temperature, approximately 22°C, to more stringent conditions at approximately 65°C. Both parameters, the salt concentration and the temperature, can be varied simultaneously, and it is also possible for one of the two



parameters to be kept constant and only the other to be varied. It is also possible to employ denaturing agents such as, for example, formamide or SDS during the hybridization. Hybridization in the presence of 50% formamide is preferably carried out at 5 42°C. Some exemplary conditions for hybridization and washing step are given below:

(1) Hybridization conditions with for example

- 10 a) 4X SSC at 65°C, or
- b) 6X SSC, 0.5% SDS, 100 µg/ml denatured fragmented salmon sperm DNA at 65°C, or
- c) 4X SSC, 50% formamide, at 42°C, or
- 15 d) 2X or 4X SSC at 50°C (low-stringency condition), or
- e) 2X or 4X SSC, 30 to 40% formamide at 42°C (low-stringency condition), or
- f) 6x SSC at 45°C, or,
- 20 g) 0.05 M sodium phosphate buffer pH 7.0, 2 mM EDTA, 1% BSA and 7% SDS.

(2) Washing steps with for example

- 25 a) 0.1X SSC at 65°C, or
- b) 0.1X SSC, 0.5% SDS at 68°C, or
- c) 0.1X SSC, 0.5% SDS, 50% formamide at 42°C, or
- d) 0.2X SSC, 0.1% SDS at 42°C, or
- 30 e) 2X SSC at 65°C (low-stringency condition), or
- f) 40 mM sodium phosphate buffer pH 7.0, 1% SDS, 2 mM EDTA.

Methods for preparing functional equivalents of the invention 35 preferably comprise the introduction of mutations into one of the promoters as shown in SEQ ID NO: 1 or 2. Mutagenesis may be random, in which case the mutagenized sequences are subsequently screened for their properties by a trial and error procedure. Particularly advantageous selection criteria comprise for example 40 the level of the resulting expression of the introduced nucleic acid sequence in a nonreproductive floral tissue.

Methods for mutagenesis of nucleic acid sequences are known to 45 the skilled worker and include by way of example the use of oligonucleotides with one or more mutations compared with the region to be mutated (e.g. in a site-specific mutagenesis). Primers with approximately 15 to approximately 75 nucleotides or

more are typically employed, with preferably about 10 to about 25 or more nucleotide residues being located on both sides of the sequence to be localized. Details and procedure for said mutagenesis methods are familiar to the skilled worker (Kunkel et al. (1987) *Methods Enzymol* 154:367-382; Tomic et al. (1990) *Nucl Acids Res* 12:1656; Upender et al. (1995) *Biotechniques* 18(1):29-30; US 4,237,224). A mutagenesis can also be achieved by treating for example transgenic expression vectors comprising one of the nucleic acid sequences of the invention with mutagenizing agents such as hydroxylamine.

An alternative possibility is to delete nonessential sequences of a promoter of the invention without significantly impairing the essential properties mentioned. Such deletion variants represent functionally equivalent fragments to the promoters described by SEQ ID NO: 1 or 2 or to functional equivalents thereof. Localization of the promoter sequence to particular essential regulatory regions can be carried out for example with the aid of a search routine to search for promoter elements. Particular promoter elements are often present in increased numbers in the regions relevant for promoter activity. This analysis can be carried out for example with computer programs such as the PLACE program ("Plant Cis-acting Regulatory DNA Elements"; Higo K et al. (1999) *Nucl Acids Res* 27(1): 297-300), the BIOBASE database "Transfac" (Biologische Datenbanken GmbH, Braunschweig; Wingender E et al. (2001) *Nucleic Acids Res* 29(1):281-3) or the PlantCARE database (Lescot M et al. (2002) *Nucleic Acids Res* 30(1):325-7).

The functionally equivalent fragments of one of the promoters of the invention - for example of the promoters described by SEQ ID NO: 1 or 2 - preferably comprise at least 200 base pair, very particularly preferably at least 500 base pairs, most preferably at least 1000 base pairs of the 3' end of the respective promoter of the invention - for example of the promoters described by SEQ ID NO: 1 or 2 - the length being calculated from the translation start ("ATG" codon) upstream in the 5' direction. Very especially preferred functionally equivalent fragments are the promoter sequences described by SEQ ID NO: 3 or 4. Further functionally equivalent fragments may be generated for example by deleting any 5'-untranslated regions still present. For this purpose, the start of transcription of the corresponding genes can be determined by methods familiar to the skilled worker (such as, for example, 5'-RACE), and the 5'-untranslated regions can be deleted by PCR-mediated methods or endonuclease digestion.

In transgenic expression cassettes of the invention, at least one of the promoters of the invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4) is functionally linked to at least one nucleic acid sequence to be expressed transgenically.

5

A functional linkage means, for example, the sequential arrangement of one of the promoters of the invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4) with a nucleic acid sequence to be expressed transgenically and, where appropriate, further genetic control sequences such as, for example, a terminator or a polyadenylation sequence in such a way that the promoter is able to fulfill its function in the transgenic expression of the nucleic acid sequence under suitable conditions, and expression of the nucleic acid sequence (i.e. transcription and, where appropriate, translation) takes place. "Suitable conditions" means in this connection preferably the presence of the expression cassette in a plant cell, preferably a plant cell comprised in a nonreproductive floral tissue of a plant.

20

Arrangements in which the nucleic acid sequence to be expressed transgenically is positioned behind one of the promoters of the invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4), so that the two sequences are covalently connected together, are preferred. In this connection, the distance between the promoter sequence and the nucleic acid sequence to be expressed transgenically is preferably less than 200 base pairs, particularly preferably less than 100 base pairs, very particularly preferably less than 50 base pairs.

30

Production of a functional linkage and production of a transgenic expression construct can be achieved by means of conventional recombination and cloning techniques as described for example in Maniatis T, Fritsch EF and Sambrook J (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY), in Silhavy TJ, Berman ML and Enquist LW (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY) and in Ausubel FM et al. (1987) Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience. However, further sequences which have for example the function of a linker with particular restriction enzyme cleavage sites or of a signal peptide may also be positioned between the two sequences. Insertion of sequences may also lead to expression of fusion proteins. It is possible and preferred for the transgenic expression construct, consisting of

45

a linkage of promoter and nucleic acid sequence to be expressed, to be integrated into a vector and be inserted into a plant genome for example by transformation.

- 5 However, an expression cassette also means constructions in which one of the promoters of the invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4) was, without necessarily having been functionally linked beforehand to a nucleic acid sequence to be expressed, introduced into a host genome, for example by targeted  
10 homologous recombination or random insertion, there undertakes regulatory control over endogenous nucleic acid sequences then functionally linked thereto, and controls the transgenic expression thereof. Insertion of the promoter – for example by a homologous recombination – in front of a nucleic acid coding for  
15 a particular polypeptide results in an expression cassette of the invention which controls the expression of the particular polypeptide selectively in the nonreproductive floral tissues. It is also possible for example for the natural promoter of an endogenous gene to be replaced by one of the promoters of the  
20 invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4), and for the expression behavior of the endogenous gene to be modified.

- A further possibility is also for the promoter to be inserted in  
25 such a way that antisense RNA to the nucleic acid coding for a particular polypeptide is expressed. In this way, expression of the particular polypeptide in the nonreproductive organs of the flower is selectively downregulated or switched off.

- 30 It is also possible analogously for a nucleic acid sequence which is to be expressed transgenically to be placed – for example by homologous recombination – behind the sequence which codes for one of the promoters of the invention (e.g. described by SEQ ID NO: 1, 2, 3 or 4), and which is located in its natural  
35 chromosomal context, so as to result in an expression cassette of the invention which controls the expression of the nucleic acid sequence to be expressed transgenically in the nonreproductive floral tissues.

- 40 The transgenic expression cassettes of the invention may comprise further genetic control sequences. The term genetic control sequences is to be understood broadly and means all sequences having an influence on the coming into existence or the function of a transgenic expression cassette of the invention. Genetic  
45 control sequences modify for example the transcription and translation in prokaryotic or eukaryotic organisms. The transgenic expression cassettes of the invention preferably

comprise as additional genetic control sequence a terminator sequence 3' downstream from the particular nucleic acid sequence to be expressed transgenically, and where appropriate further customary regulatory elements, in each case functionally linked  
5 to the nucleic acid sequence to be expressed transgenically.

Genetic control sequences also comprise further promoters, promoter elements or minimal promoters able to modify the expression-controlling properties. It is thus possible for  
10 example through genetic control sequences for tissue-specific expression to take place additionally in dependence on particular stress factors. Corresponding elements are described for example for water stress, abscisic acid (Lam E and Chua NH, J Biol Chem 1991; 266(26):17131-17135) and heat stress (Schoffl F et al.  
15 (1989) Mol Gen Genetics 217(2-3):246-53).

A further possibility is for further promoters which make transgenic expression possible in further plant tissues or in  
20 other organisms such as, for example, *E.coli* bacteria to be functionally linked to the nucleic acid sequence to be expressed. Suitable promoters are in principle all plant-specific promoters. Plant-specific promoters means in principle every promoter able to control the expression of genes, in particular foreign genes,  
25 in plants or plant parts, cells, tissues, cultures. It is moreover possible for expression to be for example constitutive, inducible or development-dependent. Preference is given to constitutive promoters, tissue-specific promoters, development-dependent promoters, chemically inducible,  
30 stress-inducible or pathogen-inducible promoters. Corresponding promoters are generally known to the skilled worker.

Further advantageous control sequences are to be found for example in the promoters of gram-positive bacteria such as amy  
35 and SPO2 or in the yeast or fungal promoters ADC1, Mfa, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH.

It is possible in principle for all natural promoters with their regulatory sequences like those mentioned above to be used for  
40 the method of the invention. It is additionally also possible for synthetic promoters to be used advantageously.

Genetic control sequences further comprise also the  
45 5'-untranslated regions, introns or noncoding 3'-region of genes such as, for example, the actin-1 intron, or the Adh1-S introns 1, 2 and 6 (generally: The Maize Handbook, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)), preferably the

genes with the gene locus At3g01980 and At1g63140 from *Arabidopsis thaliana*. It is possible to show that such regions may have a significant function in regulating gene expression. Thus, it has been shown that 5'-untranslated sequences are able  
5 to enhance the transient expression of heterologous genes. Examples of translation enhancers which may be mentioned are the 5' leader sequence from the tobacco mosaic virus (Gallie et al. (1987) Nucl Acids Res 15:8693-8711) and the like. They may in addition promote tissue specificity (Rouster J et al. (1998)  
10 Plant J 15:435-440). The nucleic acid sequences indicated in SEQ ID NO: 1, 2, 3 or 4 in each case represent the promoter region and the 5'-untranslated regions up to before the ATG start codon of the respective genes with the gene locus At3g01980 and At1g63140.

15

The transgenic expression construct may advantageously comprise one or more so-called enhancer sequences functionally linked to the promoter, which make increased transgenic expression of the nucleic acid sequence possible. Additional advantageous sequences  
20 can also be inserted at the 3' end of the nucleic acid sequences to be expressed transgenically, such as further regulatory elements or terminators. The nucleic acid sequences to be expressed transgenically may be comprised in one or more copies in the gene construct.

25

Polyadenylation signals suitable as control sequences are plant polyadenylation signals, preferably those which are essentially T-DNA polyadenylation signals from *Agrobacterium tumefaciens*. Examples of particularly suitable terminator sequences are the  
30 OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

Control sequences additionally mean those which make homologous  
35 recombination or insertion into the genome of a host organism possible or allow deletion from the genome. In homologous recombination for example the coding sequence of a particular endogenous gene can be specifically replaced by a sequence coding for a dsRNA. Methods such as cre/lox technology permit  
40 tissue-specific, and in some circumstances inducible, deletion of the transgenic expression construct from the genome of the host organism (Sauer B (1998) Methods 14(4):381-92). In this case, particular flanking sequences are attached to the target gene (lox sequences) and make later deletion by means of cre  
45 recombinase possible.

A transgenic expression cassette and/or the transgenic expression vectors derived therefrom may comprise further functional elements. The term functional element is to be understood broadly and means all elements which have an influence on the production, replication or function of the transgenic expression constructs of the invention, of the transgenic expression vectors or of the transgenic organisms. Non-restrictive examples which may be mentioned are:

- 10 a) Selection markers which confer resistance to biocides such as metabolism inhibitors (e.g. 2-deoxyglucose 6-phosphate; WO 98/45456), antibiotics (e.g. kanamycin, G 418, bleomycin, hygromycin) or – preferably – herbicides (e.g. phosphinothricin). Examples of selection markers which may be  
15 mentioned are: phosphinothricin acetyltransferases (bar and pat gene), which inactivate glutamine synthase inhibitors, 5-enolpyruvylshikimate-3-phosphate synthases (EPSP synthase genes) which confer resistance to glyphosate  
20 (N-(phosphonomethyl)glycine), glyphosate-degrading enzymes (gox gene product; glyphosate oxidoreductase), dehalogenases which for example inactivate dalapon (deh gene product), sulfonylurea- and imidazolinone-inactivating acetolactate synthases, and nitrilases which for example degrade  
25 bromoxynil (bxn gene product), the aasa gene product which confers resistance to the antibiotic apectinomycin, streptomycin phosphotransferases (SPT) which ensure resistance to streptomycin, neomycin phosphotransferases (NPTII) which confer resistance to kanamycin or geneticidin, the hygromycin phosphotransferases (HPT) which mediate  
30 resistance to hygromycin, the acetolactate synthases (ALS) which confer resistance to sulfonylurea herbicides (e.g. mutated ALS variants with, for example, the S4 and/or Hra mutation).
- 35 b) Reporter genes which code for easily quantifiable proteins and ensure via an intrinsic color or enzymic activity an assessment of the transformation efficiency or of the location or timing of the expression. Very particular  
40 preference is given in this connection to reporter proteins (Schenborn E, Groskreutz D. Mol Biotechnol. 1999; 13(1):29-44) such as the green fluorescence protein (GFP) (Sheen et al.(1995) Plant Journal 8(5):777-784), the chloramphenicol transferase, a luciferase (Ow et al. (1986) Science 234:856-859), the aequorin gene (Prasher et al.  
45 (1985) Biochem Biophys Res Commun 126(3):1259-1268), the  $\beta$ -galactosidase, with very particular preference for  $\beta$ -glucuronidase (Jefferson et al. (1987) EMBO J 6:3901-3907).

- c) Origins of replication which ensure replication of the transgenic expression constructs or transgenic expression vectors of the invention in, for example, *E. coli*. Examples which may be mentioned are ORI (origin of DNA replication),  
5 the pBR322 ori or the P15A ori (Sambrook et al.: Molecular Cloning. A Laboratory Manual, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).
- d) Elements which are necessary for agrobacterium-mediated plant  
10 transformation, such as, for example, the right or left border of the T-DNA or the vir region.

"Introduction" comprises for the purposes of the invention all  
15 methods suitable for introducing a nucleic acid sequence (for example an expression cassette of the invention) directly or indirectly into an organism (e.g. a plant) or a cell, compartment, tissue, organ or propagation material (e.g. seeds or  
20 fruits) thereof, or for generating such therein. Direct and indirect methods are comprised. The introduction can lead to a temporary (transient) presence of said nucleic acid sequence or  
25 else to a permanent (stable) presence. Introduction comprises for example methods such as transfection, transduction or transformation. The organisms used in the methods are grown or  
cultured, depending on the host organism, in the manner known to  
the skilled worker.

Introduction of a transgenic expression cassette of the invention  
into an organism or cells, tissues, organs, parts or seeds  
30 thereof (preferably into plants or plant cells, tissues, organs, parts or seeds) can advantageously be achieved by use of vectors comprising the transgenic expression cassettes. Vectors may be  
for example plasmids, cosmids, phages, viruses or else agrobacteria. The transgenic expression cassettes can be inserted  
35 into the vector (preferably a plasmid vector) via a suitable restriction cleavage site. The resulting vector can be firstly introduced and amplified in *E. coli*. Correctly transformed  
*E. coli* are selected and cultured, and the recombinant vector is isolated by methods familiar to the skilled worker. Restriction  
40 analysis and sequencing can be used to check the cloning step. Preferred vectors are those making stable integration of the expression cassette into the host genome possible.

Production of a transformed organism (or of a transformed cell or  
45 tissue) requires introduction of the appropriate DNA (e.g. the expression vector) or RNA into the appropriate host cell. A large number of methods is available for this process, which is



- referred to as transformation (or transduction or transfection) (Keown et al. (1990) *Methods in Enzymology* 185:527-537). Thus, the DNA or RNA can for example be introduced directly by microinjection or by bombardment with DNA-coated microparticles.
- 5 The cell can also be permeabilized chemically, for example with polyethylene glycol, so that the DNA is able to enter the cell by diffusion. The DNA introduction can also take place by protoplast fusion with other DNA-comprising units such as minicells, cells, lysosomes or liposomes. Electroporation is another suitable
- 10 method for introducing DNA, in which the cells are reversibly permeabilized by an electrical impulse. Corresponding methods are described (for example in Bilang et al. (1991) *Gene* 100:247-250; Scheid et al. (1991) *Mol Gen Genet* 228:104-112; Guerche et al. (1987) *Plant Science* 52:111-116; Neuhauser et al. (1987) *Theor*
- 15 *Appl Genet* 75:30-36; Klein et al. (1987) *Nature* 327:70-73; Howell et al. (1980) *Science* 208:1265; Horsch et al. (1985) *Science* 227:1229-1231; DeBlock et al. (1989) *Plant Physiology* 91:694-701; *Methods for Plant Molecular Biology* (Weissbach and Weissbach, eds.) Academic Press Inc. (1988); and *Methods in Plant Molecular*
- 20 *Biology* (Schuler and Zielinski, eds.) Academic Press Inc. (1989)).

- Vectors preferred for expression in *E. coli* are pQE70, pQE60 and pQE-9 (QIAGEN, Inc.); pBluescript vectors, Phagescript vectors,
- 25 pNH8A, pNH16a, pNH18A, pNH46A (Stratagene Cloning Systems, Inc.); pTrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia Biotech, Inc.).

- 30 Preferred vectors for expression in mammalian cells comprise pWLNE0, pSV2CAT, pOG44, pXT1 and pSG (Stratagene Inc.); pSVK3, pBPV, pMSG and pSVL (Pharmacia Biotech, Inc.). Inducible vectors which may be mentioned are pTet-tTak, pTet-Splice, pcDNA4/TO, pcDNA4/TO /LacZ, pcDNA6/TR, pcDNA4/TO/Myc-His/LacZ,
- 35 pcDNA4/TO/Myc-His A, pcDNA4/TO/Myc-His B, pcDNA4/TO/Myc-His C, pVgRXR (Invitrogen, Inc.) or the pMAM series (Clontech, Inc.; GenBank Accession No: U02443). These themselves provide the inducible regulatory control element for example for a chemically inducible expression.

- 40 Vectors for expression in yeast comprise for example pYES2, pYD1, pTEF1/Zeo, pYES2/GS, pPICZ, pGAPZ, pGAPZalpha, pPIC9, pPIC3.5, PHIL-D2, PHIL-S1, pPIC3SK, pPIC9K, and PA0815 (Invitrogen, Inc.).

Cloning vectors and techniques for genetic manipulation of ciliates and algae are known to the skilled worker (WO 98/01572; Falciatore et al. (1999) Marine Biotechnology 1(3):239-251; Dunahay et al. (1995) J Phycol 31:10004-1012).

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The methods to be used in principle for the transformation of animal cells or of yeast cells are similar to those for "direct" transformation of plant cells. Methods such as calcium phosphate or liposome-mediated transformation or else electroporation are  
10 preferred in particular.

Various methods and vectors for inserting genes into the genome of plants and for regenerating plants from plant tissues or plant  
15 cells are known (Plant Molecular Biology and Biotechnology (CRC Press, Boca Raton, Florida), Chapter 6/7, pp. 71-119 (1993); White FF (1993) Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, editors: Kung and Wu R, Academic Press, 15-38; Jenes B et al. (1993)  
20 Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, editors: Kung and R. Wu, Academic Press, pp. 128-143; Potrykus (1991) Annu Rev Plant Physiol Plant Molec Biol 42:205-225; Halford NG, Shewry PR (2000) Br Med Bull 56(1):62-73). Those mentioned above are included, for example. In  
25 the case of plants, the described methods for the transformation and regeneration of plants from plant tissues or plant cells are used for transient or stable transformation. Suitable methods are, in particular, protoplast transformation by polyethylene glycol-induced DNA uptake, calcium phosphate-mediated  
30 transformation, DEAE-dextran-mediated transformation, liposome-mediated transformation (Freeman et al. (1984) Plant Cell Physiol. 29:1353ff; US 4,536,475), biolistic methods with the gene gun ("particle bombardment" method; US 5,100,792; EP-A 0 444 882; EP-A 0 434 616; Fromm ME et al. (1990) Bio/Technology 8(9):833-9; Gordon-Kamm et al. (1990) Plant Cell 2:603),  
35 electroporation, incubation of dry embryos in DNA-containing solution, electroporation (EP-A 290 395, WO 87/06614), microinjection (WO 92/09696, WO 94/00583, EP-A 0 331 083, EP-A 0 175 966) or other methods of direct DNA introduction (DE 4 005 152, WO 90/12096, US 4,684,611). Physical methods of DNA  
40 introduction into plant cells are surveyed in Oard (1991) Biotech Adv 9:1-11.

In the case of these "direct" transformation methods, no  
45 particular requirements need be met by the plasmid used. Simple plasmids such as those of the pUC series, pBR322, M13mp series,

pACYC184 etc. can be used. If complete plants are to be regenerated from the transformed cells, it is necessary for an additional selectable marker gene to be present on the plasmid.

- 5 Besides these "direct" transformation techniques, it is also possible to carry out a transformation by bacterial infection using agrobacterium (e.g. EP 0 116 718), viral infection using viral vectors (EP 0 067 553; US 4,407,956; WO 95/34668; WO 93/03161) or using pollen (EP 0 270 356; WO 85/01856; US 10 4,684,611).

- The transformation is preferably effected using agrobacteria which comprise disarmed Ti plasmid vectors, utilizing their natural ability to transfer genes to plants (EP-A 0 270 355; EP-A 15 0 116 718).

- Agrobacterium transformation is widely used for the transformation of dicotyledons, but is also increasingly being 20 applied to monocotyledons (Toriyama et al. (1988) Bio/Technology 6: 1072-1074; Zhang et al. (1988) Plant Cell Rep 7:379-384; Zhang et al. (1988) Theor Appl Genet 76:835-840; Shimamoto et al. (1989) Nature 338:274-276; Datta et al. (1990) Bio/Technology 8: 736-740; Christou et al. (1991) Bio/Technology 9:957-962; Peng et 25 al. (1991) International Rice Research Institute, Manila, Philippines 563-574; Cao et al. (1992) Plant Cell Rep 11:585-591; Li et al. (1993) Plant Cell Rep 12:250-255; Rathore et al. (1993) Plant Mol Biol 21:871-884; Fromm et al. (1990) Bio/Technology 8:833-839; Gordon-Kamm et al. (1990) Plant Cell 2:603-618; 30 D'Halluin et al. (1992) Plant Cell 4:1495-1505; Walters et al. (1992) Plant Mol Biol 18:189-200; Koziel et al. (1993) Biotechnology 11:194-200; Vasil IK (1994) Plant Mol Biol 25:925-937; Weeks et al. (1993) Plant Physiol 102:1077-1084; Somers et al. (1992) Bio/Technology 10:1589-1594; WO 92/14828; 35 Hiei et al. (1994) Plant J 6:271-282).

- The strains mostly used for agrobacterium transformation, Agrobacterium tumefaciens or Agrobacterium rhizogenes comprise a plasmid (Ti or Ri plasmid) which is transferred to the plant 40 after agrobacterium infection. Part of this plasmid, called T-DNA (transferred DNA), is integrated into the genome of the plant cell. Alternatively, binary vectors (mini-Ti plasmids) can also be transferred into plants and integrated in the genome thereof by agrobacterium.

The use of *Agrobacterium tumefaciens* for the transformation of plants using tissue culture explants is described (inter alia Horsch RB et al. (1985) *Science* 225:1229ff.; Fraley et al. (1983) *Proc Natl Acad Sci USA* 80: 4803-4807; Bevens et al. (1983) *Nature* 5 304:184-187). Many *Agrobacterium tumefaciens* strains are able to transfer genetic material - for example the expression cassettes of the invention - such as, for example, the strains EHA101[pEHA101], EHA105[pEHA105], LBA4404[pAL4404], C58C1[pMP90] and C58C1[pGV2260] (Hood et al. (1993) *Transgenic Res* 2:208-218; 10 Hoekema et al. (1983) *Nature* 303:179-181; Koncz and Schell (1986) *Gen Genet* 204:383-396; Deblaere et al. (1985) *Nucl Acids Res* 13: 4777-4788).

On use of agrobacteria, the expression cassette must be 15 integrated into specific plasmids either into a shuttle, or intermediate, vector or into a binary vector. Binary vectors, which are able to replicate both in *E. coli* and in agrobacterium, are preferably used. They normally comprise a selection marker gene and a linker or polylinker, flanked by the right and left 20 T-DNA border sequence. They can be transformed directly into agrobacterium (Holsters et al. (1978) *Mol Gen Genet* 163:181-187). The agrobacterium acting as host organism in this case should already comprise a plasmid having the vir region. This is necessary for transfer of the T-DNA into the plant cell. An 25 agrobacterium transformed in this way can be used to transform plant cells. The use of T-DNA for transforming plant cells has been intensively investigated and described (EP-A 0 120 516; Hoekema, In: *The Binary Plant Vector System*, Offsetdrukkerij Kanters B.V., Alblasterdam, Chapter V; An et al. (1985) *EMBO J* 30 4:277-287). Various binary vectors are known, and some of them are commercially available, such as, for example, pBI101.2 or pBIN19 (Clontech Laboratories, Inc. USA; Bevan et al. (1984) *Nucl Acids Res* 12:8711), pBinAR, pPZP200 or pPTV.

35 Agrobacteria transformed with such a vector can then be used in a known manner for transforming plants, especially crop plants such as, for example, oilseed rape, by for example bathing wounded leaves or pieces of leaf in a solution of agrobacteria and then cultivating in suitable media. Transformation of plants by 40 agrobacteria is described (White FF (1993) *Vectors for Gene Transfer in Higher Plants*; in *Transgenic Plants*, Vol. 1, Engineering and Utilization, edited by SD Kung and R Wu, Academic Press, pp. 15-38; Jenes B et al. (1993) *Techniques for Gene Transfer*, in: *Transgenic Plants*, Vol. 1, Engineering and 45 Utilization, edited by S.D. Kung and R. Wu, Academic Press, pp. 128-143; Potrykus (1991) *Annu Rev Plant Physiol Plant Molec Biol* 42:205-225). Transgenic plants which have integrated the

expression systems of the invention described above can be regenerated in a known manner from the transformed cells of the wounded leaves or pieces of leaf.

- 5 Stably transformed cells (i.e. those which have integrated the introduced DNA into the DNA of the host cell) can be selected from untransformed ones if a selectable marker is a constituent of the introduced DNA. Any gene able to confer a resistance (see above) to a biocide (e.g. an antibiotic or herbicide, see above)
- 10 can act as marker, for example. Transformed cells which express such a marker gene are able to survive in the presence of concentrations of a corresponding biocide which kill an untransformed wild type. The selection marker permits the selection of transformed cells from untransformed ones (McCormick
- 15 et al. (1986) Plant Cell Reports 5:81-84). The resulting plants can be grown and crossed in the usual way. Two or more generations should be cultivated in order to ensure that the genomic integration is stable and heritable.
- 20 As soon as a transformed plant cell has been produced, it is possible to obtain a complete plant by using methods known to the skilled worker. These entail, for example, starting from callus cultures, single cells (e.g. protoplasts) or leaf disks (Vasil et
- 25 al. (1984) Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications, Academic Press; Weissbach and Weissbach (1989) Methods for Plant Molecular Biology, Academic Press). The formation of shoot and root from these still undifferentiated callus cell masses can be induced in a known manner. The resulting shoots can be planted
- 30 out and grown. Corresponding methods are described (Fennell et al. (1992) Plant Cell Rep. 11: 567-570; Stoeger et al (1995) Plant Cell Rep. 14:273-278; Jahne et al. (1994) Theor Appl Genet 89:525-533).
- 35 The effectiveness of expression of the transgenically expressed nucleic acids can be estimated for example *in vitro* by shoot-meristem propagation using one of the selection methods described above. In addition, a change in the type and level of
- 40 expression of a target gene, and the effect on the phenotype of the plant can be tested on test plants in glasshouse tests.

A further aspect of the invention relates to transgenic organisms transformed with at least one expression cassette of the

45 invention or one vector of the invention, and cells, cell

cultures, tissues, parts – such as, for example, in the case of plant organisms leaves, roots etc. – or propagation material derived from such organisms.

- 5 By organism, starting or host organisms are meant prokaryotic or eukaryotic organisms such as, for example, microorganisms or plant organisms. Preferred microorganisms are bacteria, yeasts, algae or fungi.
- 10 Preferred bacteria are bacteria of the genus *Escherichia*, *Erwinia*, *Agrobacterium*, *Flavobacterium*, *Alcaligenes*, *Pseudomonas*, *Bacillus* or cyanobacteria, for example of the genus *Synechocystis* and further bacterial genera described in Brock Biology of
- 15 Microorganisms Eighth Edition on pages A-8, A-9, A10 and A11.

- Microorganisms which are particularly preferred are those able to infect plants and thus transfer the constructs of the invention. Preferred microorganisms are those of the genus *Agrobacterium* and
- 20 especially of the species *Agrobacterium tumefaciens*. Particularly preferred microorganisms are those able to produce toxins (e.g. botulinus toxin), pigments (e.g. carotenoids or flavonoids), antibiotics (e.g. penicillin), phenylpropanoids (e.g. tocopherol), polyunsaturated fatty acids (e.g. arachidonic acid)
- 25 or vitamins (e.g. vitamin B12).

Preferred yeasts are *Candida*, *Saccharomyces*, *Hansenula* or *Pichia*.

- Preferred fungi are *Aspergillus*, *Trichoderma*, *Ashbya*, *Neurospora*,
- 30 *Fusarium*, *Beauveria* or further fungi described in Indian Chem Engr. Section B. Vol 37, No. 1,2 (1995) on page 15, table 6.

- Host or starting organisms preferred as transgenic organisms are
- 35 in particular plant organisms.

- "Plant organism or cells derived therefrom" means in general every cell, tissue, part or propagation material (such as seeds or fruits) of an organism capable of photosynthesis. Included for
- 40 the purposes of the invention are all genera and species of higher and lower plants of the plant kingdom. Annual, perennial, monocotyledonous and dicotyledonous plants are preferred.

- "Plant" means for the purposes of the invention all genera and
- 45 species of higher and lower plants of the plant kingdom. The term includes the mature plants, seeds, shoots and seedlings, and parts derived therefrom, propagation material (for example

tubers, seeds or fruits), plant organs, tissues, protoplasts, callus and other cultures, for example cell or callus cultures, and all other types of groupings of plant cells to functional or structural units. Mature plants means plants at any stage of development beyond seedling. Seedling means a young, immature plant at an early stage of development.

Plant organisms for the purposes of the invention are additionally further photosynthetically active organisms such as, for example, algae, cyanobacteria and mosses. Preferred algae are green algae, such as, for example, algae of the genus *Haematococcus*, *Phaedactylum tricornatum*, *Volvox* or *Dunaliella*. *Synechocystis*, *Chlamydomonas* and *Scenedesmus* are particularly preferred.

Particularly preferred for the purposes of the method of the invention are plant organisms selected from the group of flowering plants (Phylum Anthophyta "angiosperms"). All annual and perennial, monocotyledonous and dicotyledonous plants are comprised. The plant is preferably selected from the following plant families: *Amaranthaceae*, *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Compositae*, *Cruciferae*, *Cucurbitaceae*, *Labiatae*, *Leguminosae*, *Papilionoideae*, *Liliaceae*, *Linaceae*, *Malvaceae*, *Rosaceae*, *Rubiaceae*, *Saxifragaceae*, *Scrophulariaceae*, *Solanaceae*, *Sterculiaceae*, *Tetragoniaceae*, *Theaceae* and *Umbelliferae*.

The invention is very particularly preferably applied to dicotyledonous plant organisms. Preferred dicotyledonous plants are in particular selected from the dicotyledonous crop plants such as, for example the following

1) Category: *Dicotyledonae* (dicotyledons). Preferred families:

- *Aceraceae* (maples)

- *Cactaceae* (cacti)

- *Rosaceae* (roses, apples, almonds, strawberries)

- *Salicaceae* (willows)

## 32

- Asteraceae (compositae) especially the genus *Lactuca*, very especially the species *sativa* (lettuce), and sunflower, dandelion, *Tagetes* or *Calendula* and many others,
- 5 - Cruciferae (Brassicaceae), especially the genus *Brassica*, very especially the species *napus* (oilseed rape), *campestris* (beet), *oleracea* (e.g. cabbage, cauliflower or broccoli and other brassica species); and of the genus *Arabidopsis*, very especially the species *thaliana*, and cress, radish, canola and  
10 many others,
- Cucurbitaceae such as melon, pumpkin squash, cucumber or zucchini and many others,
- 15 - Leguminosae (Fabaceae) especially the genus *Glycine*, very especially the species *max* (soybean), soya and alfalfa, pea, beans, lupin or peanut and many others,
- 20 - Malvaceae, especially mallow, cotton, edible marshmallow, hibiscus and many others,
- Rubiaceae, preferably of the subclass Lamiidae such as, for example, *Coffea arabica* or *Coffea liberica* (coffee bush) and  
25 many others,
- Solanaceae, especially the genus *Lycopersicon*, very especially the species *esculentum* (tomato) and the genus *Solanum*, very especially the species *tuberosum* (potato) and *melongena* (eggplant) and the genus *Capsicum*, very especially the species  
30 *annuum* (paprika), and tobacco, petunia and many others,
- Sterculiaceae, preferably of the subclass Dilleniidae such as,  
35 for example, *Theobroma cacao* (cocoa bush) and many others,
- Theaceae, preferably of the subclass Dilleniidae such as, for example, *Camellia sinensis* or *Thea sinensis* (tea bush) and many  
40 others,
- Umbelliferae (Apiaceae), especially the genus *Daucus* (very especially the species *carota* (carrot), *Apium* (very especially the species *graveolens dulce* (celeriac)), and parsley and many  
45 others;



and linum, hemp, flax, spinach, carrot, sugarbeet and the various tree, nut and vine species, especially banana and kiwi fruit.

However, in addition, monocotyledonous plants are also suitable.  
5 These are preferably selected from the monocotyledonous crop plants such as, for example the families

- Arecaceae (palms)
- 10 - Bromeliaceae (pineapple, spanish moss)
- Cyperaceae (sedges)
- Liliaceae (lilies, tulips, hyacinths, onions, garlic)
- Orchidaceae (orchids)
- 15 - Poaceae (grasses, bamboos, corn, sugarcane, wheat)
- Iridaceae (buckwheat, gladioli, crocuses)

Very particular preference is given to Gramineae such as rice,  
20 corn, wheat or other cereal species such as barley, sorghum and millet, rye, triticale or oats, and the sugarcane, and all species of grasses.

Within the framework of the expression cassette of the invention,  
25 expression of a particular nucleic acid may, through a promoter having specificity for the nonreproductive organs of the flower, lead to the formation of sense RNA, antisense RNA or double-stranded RNA in the form of an inverted repeat (dsRNAi). The sense RNA can subsequently be translated into particular  
30 polypeptides. It is possible with the antisense RNA and dsRNAi to down regulate the expression of particular genes.

The method of gene regulation by means of double-stranded RNA ("double-stranded RNA interference"; dsRNAi) has been described  
35 in animal and plant organisms many times (e.g. Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A et al (1998) Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364). Express reference is made to the processes and methods described in the citations  
40 indicated.

The specificity of the expression constructs and vectors of the invention for flowers of plants is particularly advantageous. The flower has a function in attracting beneficial insects through  
45 incorporation of pigments or synthesis of volatile chemicals.

- The natural defense mechanisms of the plant, for example against pathogens, are often inadequate. Introduction of foreign genes from plants, animals or microbial sources may enhance the defenses. Examples are protection against insect damage to
- 5 tobacco through expression of the *Bacillus thuringiensis* endotoxin (Vaech et al. (1987) *Nature* 328:33-37) or protection of tobacco from fungal attack through expression of a chitinase from beans (Broglie et al. (1991) *Science* 254:1194-1197).
- 10 Cold spells during the flowering period lead to considerable crop losses every year. Targeted expression of protective proteins specifically in the flowering period may provide protection.
- 15 For such genetic engineering approaches to be highly efficient it is advantageous for there to be concentrated expression of the appropriate nucleic acid sequence to be expressed transgenically in particular in the petals of the flower. Constitutive expression in the whole plant may make the effect problematic,
- 20 for example through dilution, or impair the growth of the plant or the quality of the plant product. In addition, there may through constitutive expression be increased switching-off of the transgene ("gene silencing").
- 25 Promoters having specificity for the flower are advantageous in this connection. The skilled worker is aware of a large number of proteins whose recombinant expression in the flower is advantageous. The skilled worker is also aware of a large number of genes through which advantageous effects can likewise be
- 30 achieved through repression or switching-off thereof by means of expression of a corresponding antisense RNA. Non-restrictive examples of advantageous effects which may be mentioned are: achieving resistance to abiotic stress factors (heat, cold, drought, increased moisture, environmental toxins, UV radiation)
- 35 and biotic stress factors (pathogens, viruses, insects and diseases), improving the properties of human and animal foods, improving the growth rate or the yield, achieving a longer or earlier flowering period, altering or enhancing the scent or the coloring of the flowers. Non-restrictive examples of the nucleic
- 40 acid sequences or polypeptides which can be employed in these applications and which may be mentioned are:
1. Improved UV protection of the flowers of plants through alteration of the pigmentation through expression of
- 45 particular polypeptides such as enzymes or regulators of flavonoid biosynthesis (e.g. chalcone synthases, phenylalanine ammonia-lyases), of DNA repair (e.g.

- photolyases; Sakamoto A et al. (1998) DNA Seq 9(5-6):335-40), of isoprenoid biosynthesis (e.g. deoxyxylulose-5-phosphate synthases), of IPP synthesis or of carotenoid biosynthesis (e.g. phytoene synthases, phytoene desaturases, lycopene cyclases, hydroxylases or ketolases). Preference is given to nucleic acids which code for the *Arabidopsis thaliana* chalcone synthase (GenBank Acc. No.: M20308), the *Arabidopsis thaliana* 6-4 photolyase (GenBank Acc. No.: BAB00748) or the *Arabidopsis thaliana* blue light photoreceptor/photolyase homolog (PHH1) (GenBank Acc. No.: U62549) or functional equivalents thereof.
2. Improved protection of the flower of plants from abiotic stress factors such as drought, heat or cold, for example through overexpression of the antifreeze polypeptides (e.g. from *Myoxocephalus scorpius*; WO 00/00512), of the *Arabidopsis thaliana* transcription activator CBF1, glutamate dehydrogenases (WO 97/12983, WO 98/11240), of a late embryogenesis gene (LEA), for example from barley (WO 97/13843), calcium-dependent protein kinase genes (WO 98/26045), calcineurins (WO 99/05902), farnesyl transferases (WO 99/06580; Pei ZM et al. (1998) Science 282:287-290), ferritin (Deak M et al. (1999) Nature Biotechnology 17:192-196), oxalate oxidase (WO 99/04013; Dunwell JM (1998) Biotechnology and Genetic Engineering Reviews 15:1-32), DREB1A factor (dehydration response element B 1A; Kasuga M et al. (1999) Nature Biotechnology 17:276-286), genes of mannitol or trehalose synthesis (e.g. trehalose-phosphate synthases; trehalose-phosphate phosphatases, WO 97/42326); or through inhibition of genes such as of trehalase (WO 97/50561). Particular preference is given to nucleic acids which code for the *Arabidopsis thaliana* transcriptional activator CBF1 (Gen-Bank Acc. No.: U77378) or the antifreeze protein from *Myoxocephalus octodecemspinosus* (GenBank Acc. No.: AF306348) or functional equivalents thereof.
3. Achieving resistance for example to fungi, insects, nematodes and diseases through targeted secretion or accumulation of certain metabolites or proteins in the flower. Examples which may be mentioned are glucosinolates (nematode defense), chitinases or glucanases and other enzymes which destroy the cell wall of parasites, ribosome-inactivating proteins (RIPs) and other proteins of the plant resistance and stress response, like those induced on injury or microbial attack of plants or chemically by, for example, salicylic acid, jasmonic acid or ethylene, lysozymes from non-plant sources

- such as, for example, T4 lysozyme or lysozyme from various mammals, insecticidal proteins such as *Bacillus thuringiensis* endotoxin,  $\alpha$ -amylase inhibitor or protease inhibitors (cowpea trypsin inhibitor), glucanases, lectins (e.g. phytohemagglutinin, snowdrop lectin, wheatgerm agglutinin), RNases or ribozymes. Particular preference is given to nucleic acids which code for the chit42 endochitinase from *Trichoderma harzianum* (GenBank Acc. No.: S78423) or for the N-hydroxylating, multifunctional cytochrome P-450 (CYP79) from *Sorghum bicolor* (GenBank Acc. No.: U32624) or functional equivalents thereof.
4. Achieving defense against or attraction of insects, for example through increased release of volatile scents or messengers through, for example, enzymes of terpene biosynthesis.
5. Achieving an ability to store in flower tissues which normally comprise no storage proteins or lipids, with the aim of increasing the yield of these substances, e.g. by expression of an acetyl-CoA carboxylase or of enzymes for esterification of metabolites. Preference is given to nucleic acids which code for the *Medicago sativa* acetyl-CoA carboxylase (Accase) (GenBank Acc. No.: L25042) or functional equivalents thereof.
6. Expression of transport proteins which improve the uptake of metabolites, nutrients or water into the flower and thus optimize flower growth, metabolite composition or yield, for example through expression of an amino acid transporter which increases the rate of uptake of amino acids, or of a monosaccharide transporter which promotes the uptake of sugars. Preference is given to nucleic acids which code for the *Arabidopsis thaliana* cationic amino acid transporter (GenBank Acc. No.: X92657) or for the *Arabidopsis thaliana* monosaccharide transporter (GenBank Acc. No.: AJ002399) or functional equivalents thereof.
7. Expression of genes which bring about an accumulation of fine chemicals, such as of tocopherols, tocotrienols, phenylpropanoids, isoprenoids or carotenoids, in the flower. Examples which may be mentioned are the deoxyxylulose-5-phosphate synthases, phytoene synthases, lycopene  $\beta$ -cyclases and the  $\beta$ -carotene ketolases. Preference

is given to nucleic acids which code for the *Haematoccus pluvialis* NIES-144 (Acc. No. D45881) ketolase or functional equivalents thereof.

- 5 8. Modification of wax ester formation or of the composition of the deposited oligosaccharides to improve protection against environmental effects or to improve digestibility on use in animal or human foods. An example which may be mentioned is overexpression of endo-xyloglucan transferase. Preference is  
10 given to nucleic acids which code for the *Arabidopsis thaliana* endo-xyloglucan transferase (EXGT-A1) (Gen-Bank Acc. No.:AF163819) or functional equivalents thereof.
- 15 9. Expression of genes, DNA binding proteins, dsRNA and antisense constructions for altering the flower morphology, the time of flowering and the flower senescence, and the flower metabolism. Preference is given to constructions which increase the number of petals, e.g. through down regulation  
20 of AGAMOUS and its homologous genes (Yanofsky MF et al. (1990) Nature 346:35-39), make the time of flowering earlier, e.g. through down regulation of FLOWERING LOCUS C (FLC) (Tadege M et al. (2001) Plant J 28(5):545-53) or later, e.g. through overexpression of FLC, and delay senescence, e.g.  
25 through conferring a flower-specific ethylene insensitivity.
10. Generation of sterile plants by preventing pollination and/or germination by means of the expression of a suitable inhibitor, for example of a toxin, in flowers.
- 30 11. Production of nutraceuticals such as, for example
  - a) carotenoids and/or phenylpropanoids e.g. through  
35 optimization of the flowers' own metabolic pathways, e.g. through expression of enzymes and regulators of isoprenoid biosynthesis. Preference is given to nucleic acids which code for the *Arabidopsis thaliana* chalcone synthase (GenBank Acc. No.: M20308), the *Arabidopsis thaliana* 6-4 photolyase (GenBank Acc.No.:BAB00748) or the  
40 *Arabidopsis thaliana* blue light photoreceptor/photolyase homolog (PHH1) (GenBank Acc. No.: U62549) or functional equivalents thereof. Preference is likewise given to nucleic acids which code for enzymes and regulators of isoprenoid biosynthesis such as the  
45 deoxyxylulose-5-phosphate synthases and of carotenoid biosynthesis such as the phytoene synthases, lycopene cyclases and ketolases, such as of tocopherols,

- tocotrienols, phenylpropanoids, isoprenoids or carotenoids, in the flower. Examples which may be mentioned are the deoxyxylulose-5-phosphate synthases, phytoene synthases, lycopene cyclases and the carotene ketolases. Particular preference is given to nucleic acids which code for the *Haematoccus pluvialis*, NIES-144 (Acc. No. D45881) ketolase or functional equivalents.
- 5
- b) polyunsaturated fatty acids such as, for example, arachidonic acid or EPA (eicosapentaenoic acid) or DHA (docosahexaenoic acid) through expression of fatty acid elongases and/or desaturases or production of proteins having improved nutritional value, such as, for example, having a high content of essential amino acids (e.g. the methionine-rich 2S albumin gene of the Brazil nut). Preference is given to nucleic acids which code for the *Bertholletia excelsa* methionine-rich 2S albumin (GenBank Acc. No.: AB044391), the *Physcomitrella patens*  $\Delta 6$ -acyl lipid desaturase (GenBank Acc. No.: AJ222980; Girke et al. (1998) Plant J 15:39-48), the *Mortierella alpina*  $\Delta 6$ -desaturase (Sakura-dani et al 1999 Gene 238:445-453), the *Caenorhabditis elegans*  $\Delta 5$ -desaturase (Michaelson et al. (1998) FEBS Letters 439:215-218), the *Caenorhabditis elegans*  $\Delta 5$ -fatty-acid desaturase (des-5) (GenBank Acc. No.: AF078796), the *Mortierella alpina*  $\Delta 5$ -desaturase (Michaelson et al. J Biol Chem 273:19055-19059), the *Caenorhabditis elegans*  $\Delta 6$ -elongase (Beaudoin et al. (2000) Proc Natl. Acad. Sci. 97:6421-6426), the *Physcomitrella patens*  $\Delta 6$ -elongase (Zank et al. (2000,) Biochemical Society Transactions 28:654-657) or functional equivalents thereof.
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12. Production of pharmaceuticals such as, for example, antibodies, vaccines, hormones and/or antibiotics as described, for example, in Hood EE & Jilka JM (1999) Curr Opin Biotechnol 10(4):382-6; Ma JK & Vine ND (1999) CurrTop Microbiol Immunol 236:275-92.
- 35
- 40 Further examples of advantageous genes are mentioned for example in Dunwell JM (2000) Transgenic approaches to crop improvement. J Exp Bot. 51 Spec No:487-96.
- A further aspect of the invention relates to the use of the transgenic organisms of the invention described above, and of the cells, cell cultures, parts – such as, for example, in the case of transgenic plant organisms roots, leaves etc. – and transgenic
- 45

propagation material such as seeds or fruits, derived therefrom for producing human or animal foods, pharmaceuticals or fine chemicals.

- 5 Preference is further given to a method for the recombinant production of pharmaceuticals or fine chemicals in host organisms, where a host organism is transformed with one of the expression cassettes described above, and this expression cassette comprises one or more structural genes which code for
- 10 the desired fine chemical, or catalyze the biosynthesis thereof, the transformed host organism is cultivated, and the desired fine chemical is isolated from the cultivation medium. This method can be applied widely to fine chemicals such as enzymes, vitamins, amino acids, sugars, fatty acids, natural and synthetic
- 15 flavorings, aromatizing substances and colorants. Production of tocopherols and tocotrienols, and carotenoids such as, for example, astaxanthin is particularly preferred. Cultivation of the transformed host organisms and isolation from the host organisms or from the cultivation medium take place by methods
- 20 known to the skilled worker. The production of pharmaceuticals such as, for example, antibodies or vaccines is described in Hood EE & Jilka JM (1999) Curr Opin Biotechnol 10 (4)382-6; Ma JK & Vine ND (1999) Curr Top Microbiol Immunol 236:275-92.

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## Sequences

1. SEQ ID NO: 1 2051bp fragment of promoter (and if appropriate 5' untranslated region of the Arabidopsis thaliana gene locus At3g01980 (76L promoter)  
5
2. SEQ ID NO: 2 2192bp fragment of promoter (and if appropriate 5' untranslated region of the Arabidopsis thaliana gene locus At1g63140 (84L promoter)  
10
3. SEQ ID NO: 3 Functionally equivalent fragment (1045 bp) of promoter (and if appropriate 5' untranslated region of Arabidopsis thaliana gene locus At3g01980 (76S promoter)  
15
4. SEQ ID NO: 4 Functionally equivalent fragment (1109 bp) of promoter (and if appropriate 5' untranslated region of Arabidopsis thaliana gene locus At1g63140 (84L promoter)  
20
5. Seq ID No: 5 Oligonucleotide primer 76sSmaI  
25 5'-CCCGGGTGCCAAAGTAACTCTTTAT-3'
6. Seq ID No: 6 Oligonucleotide primer 76assSali  
5'-GTCGACAGGTGCATGACCAAGTAAC-3'
7. Seq ID No: 7 Oligonucleotide primer 76aslSali  
30 5'-GTCGACTATCCTCTGCGCAATGAAT-3'
8. Seq ID No: 8 Oligonucleotide primer 84sSmaI  
35 5'-CCCGGGAAATCGAGAAAGATAGGTA-3'
9. Seq ID No; 9 Oligonucleotide primer 84assSali  
5'-GTCGACAAAGGGTTATAGGAGACTG-3'
10. Seq ID No: 10 Oligonucleotide primer 84aslSali  
40 5'-GTCGACCATGTTTCAGAGGATATGT-3'
11. SEQ ID NO: 11 Nucleic acid sequence (cDNA) encoding the gene product of the Arabidopsis thaliana gene locus At3g01980  
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12. SEQ ID NO: 12 Amino acid sequence encoding the gene product of the Arabidopsis thaliana gene locus At3g01980
- 5 13. SEQ ID NO: 13 Nucleic acid sequence (cDNA) encoding the gene product of the Arabidopsis thaliana gene locus At1g63140
- 10 14. SEQ ID NO: 14 Amino acid sequence encoding the gene product of the Arabidopsis thaliana gene locus At1g63140
- 15 15. SEQ ID NO: 15 Nucleic acid sequence (cDNA) encoding the oilseed rape homolog (H2) of the At3g01980 gene product
- 20 16. SEQ ID NO: 16 Amino acid sequence encoding the oilseed rape homolog (H2) of the At3g01980 gene product
- 25 17. SEQ ID NO: 17 Nucleic acid sequence (cDNA) encoding the oilseed rape homolog (H3) of the At3g01980 gene product
- 30 18. SEQ ID NO: 18 Amino acid sequence encoding the oilseed rape homolog (H3) of the At3g01980 gene product
- 35 19. SEQ ID NO: 19 Nucleic acid sequence (cDNA) encoding the oilseed rape homolog (H4) of the At1g63140 gene product
- 40 20. SEQ ID NO: 20 Amino acid sequence encoding the oilseed rape homolog (H4) of the At1g63140 gene product
- 45 21. SEQ ID NO: 21 Nucleic acid sequence (cDNA) encoding the oilseed rape homolog (H5) of the At1g63140 gene product
22. SEQ ID NO: 22 Amino acid sequence encoding the oilseed rape homolog (H5) of the At1g63140 gene product

23.-32 SEQ ID NO: 23 bis 32: Sequence motifs for proteins with specific expression in the nonreproductive floral tissues.

- 5 33. Seq ID No: 33 Oligonucleotide primer GUS for  
5'-cac ttt tcc cgg caa taa cat-3'
34. Seq ID No: 34 Oligonucleotide primer GUS rev  
5'-atc agg aag tga tgg agc atc-3'
- 10 35. Seq ID No: 35 Oligonucleotide primer TUB for  
5'-gac cct gtc cca cct cca a-3'
- 15 36. Seq ID No: 36 Oligonucleotide primer TUB rev  
5'-tga gaa ctg cga ttg ttt gca-3'

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## Figures

The general abbreviations used in the following figures have the following meaning:

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GUS:	reporter gene (bacterial $\beta$ -glucuronidase)
Int:	Intron
NosT:	nopaline synthase (NOS) terminator sequence
NptII:	BASTA resistance
NosP:	nopaline synthase (NOS) promoter sequence
AadA:	bacterial spectinomycin resistance

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1. Fig. 1: Diagrammatic representation of the vector pSUN3-76L-GUS. Further abbreviations have the following meaning:

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76L: 76L promoter of SEQ ID NO:1

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2. Fig. 2: Diagrammatic representation of the vector pSUN3-76S-GUS. Further abbreviations have the following meaning:

76S: 76S promoter of SEQ ID NO: 3

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3. Fig. 3: Diagrammatic representation of the vector pSUN3-84L-GUS. Further abbreviations have the following meaning:

84L: 84L promoter of SEQ ID NO: 2

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4. Fig. 4: Diagrammatic representation of the vector pSUN3-84S-GUS. Further abbreviations have the following meaning:

84S: 84S promoter of SEQ ID NO: 4

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5. Fig. 5: Diagrammatic representation of the vector pSUN5-P76-GUS. Further abbreviations have the following meaning:

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P76: 76S promoter of SEQ ID NO: 3

6. Fig.: 6: The expression patterns of the promoters 76 (A) and 84 (B) in the flower of *Arabidopsis thaliana* are shown. White/pale gray areas indicate tissues without promoter activity.
- 5
7. Fig.: 7: The expression patterns of the promoters 76 (A) and 84 (B) in the inflorescences and leaves of *Arabidopsis thaliana* are shown. White/pale gray areas indicate tissues without promoter activity.
- 10
8. Fig. 8: The resolution of the promoter activity of the promoter 76 over time during the floral development of *Arabidopsis thaliana* is shown. A:  $\beta$ -glucuronidase mRNA quantities in six stages of the floral development (P1 to P6) of *Arabidopsis thaliana* for the promoter 76. The data were determined by means of quantitative "real time" PCR and standardized with the 1 kb P76 promoter during flowering stage P4 (to this end, the value in question was set equal to 1). B: The points in time of the development of the *Arabidopsis* flowers which correspond to the flowering stages P1 to P6 are shown.
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9. Fig. 9: The resolution of the promoter activity of the promoter 84 over time during the floral development of *Arabidopsis thaliana* is shown. A:  $\beta$ -glucuronidase mRNA quantities in six stages of the floral development (P1 to P6) of *Arabidopsis thaliana* for the promoter 84. the data were determined by means of quantitative "real time" PCR and standardized with the during flowering stage P2 (to this end, the value in question was set equal to 1). B: The points in time of the development of the *Arabidopsis* flowers which correspond to the flowering stages P1 to P6 are shown.
- 25
- 30
- 35 10. Fig.10: A resolution of the promoter activity of the promoter 76 over time during the floral development of *Tagetes erecta* are shown. A:  $\beta$ -glucuronidase enzyme activity (shown in pmol of methyl umbelliferon/min/mg protein) during six stages of the floral development (P1 to P6) of *Tagetes erecta* for the promoter 76. In each case 3 individual measurements are shown (black bars, gray bars, white bars).
- 40
- 45
- B: The points in time of the development of the *Tagetes* flowers which correspond to the flowering stages P1 to P6 are shown.

11. Fig. 11: The resolution of the promoter activity of the promoter 76 over time during the floral development of *Tagetes erecta* is shown. A:  $\beta$ -glucuronidase mRNA quantities in six stages of the floral development (P1 to P6) of *Tagetes erecta* for the promoter 76. The data were determined by means of quantitative "real time" PCR and standardized with the during flowering stage P4 (to this end, the value in question was set equal to 1). B: The points in time of the development of the *Tagetes* flowers which correspond to the flowering stages P1 to P6 are shown.
12. Fig. 12: Protein sequence alignment between the SEQ ID NO: 12 amino acid sequence (cDNA) encoding the gene product of the *Arabidopsis thaliana* gene locus At3g01980 and a cDNA clone from a *Brassica napus* floral cDNA library.
13. Fig. 13: Protein sequence alignment between the SEQ ID NO: 14 amino acid sequence (cDNA) encoding the gene product of the *Arabidopsis thaliana* gene locus At1g63140 and a cDNA clone from a *Brassica napus* floral cDNA library.
14. Fig. 14: diagrammatic representation of the inverse PCR ("iPCR"). For the "iPCR", genomic DNA of a target organism having the promoter sequence to be isolated is completely digested with a given restriction enzyme, and then the individual fragments are religated, i.e. connected together to form a circular molecule, in a diluted mixture. The large number of resulting circular DNA molecules includes those comprising the known sequence (i.e. the sequence coding for a homologous protein). The circular molecule can be amplified, starting therefrom, by means of PCR using a primer pair in which both primers are able to anneal to the known sequence segment. Abbreviations: P – promoter sequence; CR – coding region; L – ligation site; PCR – polymerase chain reaction. Arrows represent the binding site of potential oligonucleotide primers in the area of the coding region.

#### Examples

#### General methods:

Oligonucleotides can be chemically synthesized for example in a known manner by the phosphoramidite method (Voet & Voet (1995), 2<sup>nd</sup> edition, Wiley Press New York, pages 896-897). The cloning steps carried out for the purposes of the present invention, such as, for example, restriction cleavages, agarose gel

electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of *E. coli* cells, culturing of bacteria, replication of phages and sequence analysis of recombinant DNA, are carried out as described in Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6. Recombinant DNA molecules are sequenced by the method of Sanger (Sanger et al. (1977) *Proc Natl Acad Sci USA* 74:5463-5467) using an ABI laser fluorescence DNA sequencer.

10

To generate transgenic *Arabidopsis* plants, *Agrobacterium tumefaciens* (strain C58C1 pMP90) is transformed with various promoter GUS vector constructs. The agrobacterial strains are subsequently used for the generation of transgenic plants. To this end, an individual transformed *Agrobacterium* colony is incubated in a 4 ml culture (medium: YEB medium supplemented with 50 µg/ml kanamycin and 25 µg/ml of rifampicin overnight at 28°C. This culture is subsequently used to inoculate a 400 ml culture in the same medium, and this culture is incubated overnight (28°C, 220 rpm) and spun down (GSA rotor, 8000rpm, 20 min). The pellet is resuspended in infiltration medium (1/2 MS medium; 0.5 g/l MES, pH 5.8; 50 g/l sucrose). The suspension is introduced into a plant box (Duchefa) and 100 ml of SILVET L-77 (polyalkylene oxide-modified heptamethyltrisiloxane; Osi Specialties Inc., Cat. P030196) was added to a final concentration of 0.02%. In a desiccator, the plant box together with 8 to 12 plants is exposed to a vacuum for 10 to 15 minutes, followed by spontaneous aeration. This is repeated 2 to 3 times. Thereafter, all the plants are planted into plant pots containing moist soil and grown under long-day conditions (illumination for 16 hours) (daytime temperature 22 to 24°C, nighttime temperature 19°C; relative atmospheric humidity 65%). The seeds are harvested after 6 weeks.

35 Example 1: Growth conditions of the plants for tissue-specific RT-PCR analysis

To obtain 4- or 7-day-old seedlings, in each case approximately 400 seeds (*Arabidopsis thaliana* ecotype Columbia) are surface-sterilized for 2 minutes with an 80% strength ethanol solution, treated for 5 minutes with sodium hypochlorite solution (0.5% v/v), washed three times with distilled water and incubated at 4°C for 4 days to ensure uniform germination. Thereafter, the seeds are incubated on Petri dishes comprising MS medium (Sigma M5519) with addition of 1% sucrose, 0.5 g/l MES (Sigma M8652), 0.8% Difco-Bacto agar (Difco 0140-01), pH 5.7. The seedlings are grown in a 16-hour-light/8-hour-dark photoperiod (Philips 58W/33

white-light lamps) at 22°C and harvested after 4 and 7 days, respectively, after the beginning of the germination phase.

- To obtain roots, 100 seeds are sterilized as described above, 5 incubated for 4 days at 4°C and then grown in 250 ml flasks comprising MS medium (Sigma M5519) with addition of a further 3% sucrose and 0.5 g/l MES (Sigma M8652), pH 5.7. The seedlings are grown in a 16-hour-light/8-hour-dark photoperiod (Philips 58W/33 white-light lamps) at 22°C, 120 rpm, and harvested after 3 weeks. 10 For all other plant organs which are used, the seeds are sown on standard soil (type VM, Manna-Italia, Via S. Giacomo 42, 39050 San Giacomo/ Laives, Bolzano, Italy), incubated for 4 days at 4°C to ensure uniform germination and then grown in a 16-hour-light/8-hour-dark photoperiod (OSRAM Lumi-lux Daylight 15 36W/12 fluorescent tubes) at 22°C. Young rosette leaves are harvested in the 8-leaf stage (after 3 weeks), and mature rosette leaves are harvested after 8 weeks shortly before stems are formed. Inflorescences (apices) of the shooting stems are harvested shortly after shooting. Stems, stem leaves and flower 20 buds are harvested at developmental stage 12 (Bowmann J (ed.), Arabidopsis, Atlas of Morphology, Springer New York, 1995) prior to stamen development. Opened flowers are harvested at stage 14 immediately after stamen development. Wilting flowers are harvested at stage 15 to 16. The green and yellow pods which were 25 used were 10 to 13 mm in length.

#### Example 2: RNA extraction and cDNA synthesis

- Total RNA is isolated from the plant organs described in Example 30 1 at various points in time of the development, as described (Prescott A, Martin C (1987) Plant Mol Biol Rep 4:219-224). The reverse-transcriptase polymerase chain reaction (RT-PCR) is used to detect the cDNA of the gene transcripts of At3g01980 and At1G63140. Prior to cDNA synthesis, all RNA samples are treated 35 with DNaseI (15 units, Boehringer, Mannheim). The first-strand cDNA synthesis is carried out starting from 6 µg of total RNA with an oligo-(dT) primer and RT Superscript™II enzyme (300 units) following the manufacturer's instructions in a total volume of 20 µl (Life Technologies, Gaithersburg, MD). To this 40 end, 150 ng of "Random Hexamer Primer" are added in a final volume of 12 µl. The mixture is heated for 10 minutes at 70°C and subsequently immediately cooled on ice. Then, 4 µl of the 5X first-strand buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP-mix (in each case 10 mM dATP, dCTP, dGTP and dTTP) and RNase 45 inhibitor (5 units, Böhringer Mannheim) are added. The mixture is heated for 2 minutes at 42°C, RT Superscript™II enzyme

(300 units, Life Technologies) is added, and the mixture is incubated for 50 minutes at 42°C.

### Example 3: Detection of the tissue-specific expression

- 5 To determine the properties of the promoter and to identify the essential elements thereof, which account for its tissue specificity, it is necessary to place the promoter itself and various fragments thereof before what is known as a reporter
- 10 gene, which makes possible a determination of the expression activity. An example which may be mentioned is the bacterial  $\beta$ -glucuronidase (Jefferson et al. (1987) EMBO J 6:3901-3907). The  $\beta$ -glucuronidase activity can be determined *in planta* by means of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl- $\beta$ -D-
- 15 glucuronic acid in an activity stain (Jefferson et al. (1987) Plant Mol Biol Rep 5:387-405). To study the tissue specificity, the plant tissue is dissected, embedded, stained and analyzed as described (for example Bäumlein H et al. (1991) Mol Gen Genet 225:121-128).
- 20 MUG (methylumbelliferylglucuronide) is used as substrate for the quantitative determination of the  $\beta$ -glucuronidase activity; it is cleaved into MU (methylumbelliferone) and glucuronic acid. Under alkaline conditions, this cleavage can be monitored
- 25 quantitatively by fluorometry (excitation at 365 nm, measurement of the emission at 455 nm; SpectroFluorimeter Thermo Life Sciences Fluoroscan) as described (Bustos MM et al. (1989) Plant Cell 1:839-853).

### 30 Example 4: Cloning the promoters

- To isolate the complete promoters of Seq ID NO: 1 or 2, genomic DNA is extracted from *Arabidopsis thaliana* (ectotype Landsberg erecta) as described (Galbiati M et al. Funct Integr Genomics
- 35 2000, 20 1:25-34). The isolated DNA is employed as template DNA in a PCR, using the following primers:

Promoter	Forward primer	Reverse primer
40 761 (SEQ ID NO:2)	SEQ ID NO: 5 (76s)	SEQ ID NO: 7 (76asl)
841 (SEQ ID NO:3)	SEQ ID NO: 8 (84s)	SEQ ID NO: 10 (84asl)
76s (SEQ ID NO:4)	SEQ ID NO: 5 (76s)	SEQ ID NO: 6 (76ass)
84s (SEQ ID NO:5)	SEQ ID NO: 8 (84s)	SEQ ID NO: 9 (84ass)

- 45 The amplification is carried out as follows:

80 ng genomic DNA  
1X Expand™ Long Template PCR buffer



- 2.5 mM MgCl<sub>2</sub>,  
 350 µM of each dATP, dCTP, dGTP and dTTP  
 300 nM of each primer -(SEQ ID NO: 5 and 7 for promoter 761  
 and SEQ ID NO 8 and 10 for promoter 84s)  
 5 2.5 units Expand™ Long Template polymerase (Roche  
 Diagnostics).  
 in a final volume of 25 µl

The following temperature program is used (PTC-100TM model QfiV;  
 10 MJ Research, Inc., Watertown, Massachusetts):

- 1 cycle with 120 seconds at 94°C  
 35 cycles with 10 seconds at 94°C, 30 seconds at 55°C and  
 3 minutes at 68°C.  
 15 1 cycle for 30 minutes 45 at 68°C

The PCR products were cleaved with the restriction endonucleases  
 SmaI and SalI and cloned into the vector pSUN::GUS. The resulting  
 constructs are pSUN3-76L::GUS (Fig.9), pSUN3-76S::GUS (Fig.10),  
 20 pSUN3-84L::GUS (Fig.11) and pSUN3-84S::GUS (Fig.11). After these  
 constructs have been stably transformed into *Arabidopsis*  
*thaliana*, RNA can be obtained from the various tissues, and the  
 expression of the GUS gene can be shown qualitatively by RT-PCR  
 and quantitatively by means of "Real Time" PCR.

25 The method for the quantitative "Real Time" PCR is described for  
 example in Bustin SA (2000) J Mol Endocrinol 25(2):169-93.

The primers

- 30 GUS for 5'-cac ttt tcc cgg caa taa cat-3'  
 GUS rev 5'-atc agg aag tga tgg agc atc-3'

were used for detecting and quantifying the GUS mRNA. The values  
 35 were standardized with the constitutively expressed tubulin. To  
 detect and quantify tubulin, the primers

- TUB for 5'-gac cct gtc cca cct cca a-3'  
 TUB rev 5'-tga gaa ctg cga ttg ttt gca-3'

40 were used.

Example 5: TAIL-PCR

45 The "TAIL-PCR" is carried out in accordance with an adapted  
 protocol of the method of Liu et al. (1995) Plant J 8(3):457-463  
 and Tsugeki et al. (1996) Plant J 10(3):479-489 (cf. Fig. 9). The

## 50

following mastermix (quantities per reaction mix) is employed for a first PCR reaction:

- 11 µl steril H<sub>2</sub>O (double-distilled)
- 5     2 µl primer stock solution of the specific primer 1 (5mM)
- 3 µl AD2 primer stock solution (20mM)
- 2 µl 10x PCR buffer
- 2 µl 10x dNTP
- 0.2 µl Taq polymerase

10

In a PCR vessel, 19 µl of this mastermix are pipetted to 1 µl of a preparation of genomic DNA of the target organism in question (preparation as described by Galbiati M et al. (2000) Funct Integr Genomics 20(1):25-34)) and mixed thoroughly by pipetting.

15

The primary PCR reaction is carried out under the following conditions:

- 94°C for 1 minute
- 20     - four cycles with 94°C for 10 seconds, 62°C for 1 minute and 72°C for 150 seconds
- 94°C for 10 seconds, 25°C for 3 minutes, 0.2°C/s to 72°C and
- 25     72°C for 150 seconds
- fourteen cycles at 94°C for 10 seconds, 69°C for 1 minute, 72°C for 150 seconds, 94°C for 10 seconds, 68°C for 1 minute, 72°C for 150 seconds, 94°C for 10 seconds, 44°C for 1 minute
- 30     and 72°C for 150 seconds
- 72°C for 5 minutes, then 4°C until further use.

The product of the PCR reaction is diluted 1:50, and 1 µl of each  
35 diluted sample is used for a second PCR reaction (secondary PCR). The following mastermix is employed for this purpose (quantities per reaction mix):

- 12 µl of sterile H<sub>2</sub>O (double distilled)
- 40     2 µl 10x PCR buffer (1.5 mM MgCl<sub>2</sub>)
- 2 µl 10x dNTP
- 2 µl primer stock solution of the specific primer 2 (5 mM)
- 2 µl AD2 primer stock solution
- 0.2µl Taq polymerase

45

## 51

In each case 20.2 µl of the second mastermix are added to in each case 1 µl of the 1:50 diluted primary PCR product, and the secondary PCR is carried out under the following conditions:

- 11 cycles at 94°C for 10 seconds, 64°C for 1 minute, 72°C for 150 seconds, 94°C for 10 seconds, 64°C for 1 minute, 72°C for 150 seconds, 94°C for 10 seconds, 44°C for 1 minute, 72°C for 150 seconds,
- 72°C for 5 minutes, then 4°C until further use.

10

The product of the PCR reaction is diluted 1:10, and 1 µl of each diluted sample is used for a third PCR reaction (tertiary PCR). The following mastermix is employed for this purpose (quantities per reaction mix):

15

- 18 µl of sterile H<sub>2</sub>O (double distilled)
- 3 µl 10x PCR buffer (1.5 mM MgCl<sub>2</sub>)
- 3 µl 10x dNTP
- 3 µl primer stock solution of the specific primer 3 (5 mM)
- 3 µl AD2 primer stock solution
- 0.5 µl Taq polymerase

20

In each case 30.3 µl of this mastermix are added to in each case 1 µl of the 1:10 diluted secondary PCR product, and the tertiary PCR is carried out under the following conditions:

25

- 19 cycles at 94°C for 15 seconds, 44°C for 1 minute, 72°C for 150 seconds,
- 72°C for 5 minutes, then 4°C until further use.

30

In each case 5 µl of the products of the PCR 1, 2 and 3 of each sample are separated on a 2% strength agarose gel. Those PCR products which, owing to the treated specific primers, show the expected size decrement are, if necessary, purified from the gel, reamplified with the primer pair which was used last, and then sequenced.

35

## Reagents:

- 40 Taq polymerase 5U/µl
- 10x PCR buffer (1.5 mM MgCl<sub>2</sub>)
- 10x dNTP stock solution: 2 mM

## Primers:

- 45 Degenerate random primer (stock solutions 20 µM):
- AD1: 5'-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3'
- AD2: 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3'

AD5: 5'-(A/T)CAGNTG(A/T)TNGTNCTG-3'

Example 6: Inverse PCR (iPCR) for the amplification of insert-flanking DNA

5

The "iPCR" is carried out in accordance with an adapted protocol of the method of Long et al.(1993) PNAS 90:10370 (cf. Fig.8):

- 10      1. Restriction of approx. 2 µg of genomic DNA with *Bst*YI for approximately 2 hours at 37°C in a total volume of 50 µl.
2. Ligation of 25 µl of the restriction mix with 3U T4-DNA ligase at 15°C overnight in a total volume of 300 µl.
- 15      3. Phenol/chloroform extraction and subsequent chloroform extraction of the ligation mix. After ethanol precipitation, take up DNA in 10 µl of sterile H<sub>2</sub>O (double-distilled).
- 20      4. Employ 2.5 µl of the DNA solution for the PCR

Reaction mix:

- 25      2.5 µl of the DNA solution
- 10 µl 10x PCR buffer
- 2 µl dNTP (mixture of 10 mM each)
- 5 µl primer 1 (25pmol)
- 5 µl primer 2 (25pmol)
- 1,5 µl Taq polymerase
- 30      74 µl H<sub>2</sub>O (double-distilled, sterile)
- to a total volume of 100 µl

- 35      PCR protocol: 4 minutes at 94°C. Then 35 cycles with 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C. Finally, 8 minutes at 72°C, then 4°C until further use.

The PCR product is checked by gel electrophoresis, purified and subsequently sequenced as PCR product.

- 40      Example 6: Production of transgenic *Tagetes* plants

- Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige & Skoog (1962) *Physiol Plant* 15:473-497; pH 5.8, 2% sucrose). Germination takes place in a
- 45      temperature/light/time interval of 18 to 28°C/20 to 200 µE/3 to 16 weeks, but preferably at 21°C, 20 to 70 µE, for 4 to 8 weeks.

## 53

All leaves of the plants which have developed in vitro by then are harvested and cut transverse to the central vein. The leaf explants resulting therefrom, with a size of 10 to 60 mm<sup>2</sup>, are stored during the preparation in liquid MS medium at room temperature for not more than 2 h.

Any *Agrobacterium tumefaciens* strain, but preferably a supervirulent strain such as, for example, EHA105 with an appropriate binary plasmid, which may harbor a selection marker gene (preferably bar or pat) and one or more trait or reporter genes (for example pS5KETO2 and pS5AP3PKETO2), is cultivated overnight and used for cocultivation with the leaf material. The bacterial strain can be cultured as follows: a single colony of the appropriate strain is inoculated in YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate x 7 H<sub>2</sub>O) with 25 mg/l kanamycin and cultured at 28°C for 16 to 20 h. The bacterial suspension is then harvested by centrifugation at 6000 g for 10 min, and resuspended in liquid MS medium so as to result in an OD<sub>600</sub> of about 0.1 to 0.8. This suspension is used for the cocultivation together with the leaf material.

Immediately before the cocultivation, the MS medium in which the leaves have been stored is replaced by the bacterial suspension. Incubation of the leaves in the agrobacterial suspension took place at room temperature with gentle shaking for 30 min. The infected explants are then put on an MS medium solidified with agar (e.g. 0.8% plant agar (Duchefa, NL)), with growth regulators such as, for example, 3 mg/l benzylaminopurine (BAP) and 1 mg/l indolylacetic acid (IAA). The orientation of the leaves on the medium is immaterial. Cultivation of the explants takes place for 1 to 8 days, but preferably for 6 days, during which the following conditions can be used: light intensity: 30 to 80 µmol/m<sup>2</sup> x sec, temperature: 22 to 24°C, 16/18-hour photoperiod. The cocultivated explants are then transferred to fresh MS medium, preferably with the same growth regulators, this second medium additionally containing an antibiotic to suppress bacterial growth. Timentin in a concentration of 200 to 500 mg/l is very suitable for this purpose. The second selective component employed is one for selecting for successful transformation. Phosphinothricin in a concentration of 1 to 5 mg/l selects very efficiently, but other selective components according to the method to be used are also conceivable.

After one to three weeks in each case, the explants are transferred to fresh medium until plumules and small shoots develop, which are then transferred to the same basal medium including timentin and PPT or alternative components with growth regulators, namely, for example, 0.5 mg/l indolylbutyric acid (IBA) and 0.5 mg/l gibberellic acid GA3, for rooting. Rooted shoots can be transferred into the glasshouse.

In addition to the method described, the following advantageous modifications are possible:

- Before the explants are infected with the bacteria, they can be preincubated on the medium described above for the cocultivation for 1 to 12 days, preferably 3 to 4. This is followed by infection, cocultivation and selective regeneration as described above.
- The pH for the regeneration (normally 5.8) can be lowered to pH 5.2. This improves control of the growth of agrobacteria.
- Addition of  $\text{AgNO}_3$  (3 to 10 mg/l) to the regeneration medium improves the condition of the culture, including the regeneration itself.
- Components which reduce phenol formation and are known to the skilled worker, such as, for example citric acid, ascorbic acid, PVP and many others, have beneficial effects on the culture.
- Liquid culture medium can also be used for the whole method. The culture can also be incubated on commercially available supports which are positioned on the liquid medium.